Post-transcriptional regulation of gene expression during male gametogenesis

Regulatory and structural properties of the 5'-untranslated region of pollen-expressed genes

Raymond Hulzink

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Regulatory and structural properties of the 5'-untranslated region of pollen-expressed genes

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Voor mijn ouders en Ilse

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Chapter 1

General introduction

Sexual reproduction in angiosperms

It was the era of the large dinosaurs and the small mammals (the Cretaceous period), when the first angiosperms or flowering plants made their appearance on earth. Despite the appetite of a large and diverse community of herbivorous dinosaurs and mammals, a high ecologic density of the gymnosperm flora and a biotic crisis as a result of a meteorite impact on earth, the middle of the Cretaceous Period (about 100 million years ago) was the start of a tremendous adaptive radiation of flowering plants which led to the development of more than 250,000 species nowadays. The evolutionary expansion was facilitated through adaptations as closed carpels (ovule-bearing structures) and double fertilization. Closed carpels allow fertilization to occur in a "conditioned" environment and therefore enable a selective screening of compatible versus incompatible male gametophytes and exclusion of unwanted pathogens and insects. Double fertilization is a prerequisite for rapid synthesis and storage of nutrients in seeds and, therefore, for a fast and frequent release of progeny.

One of the most striking features of the evolutionary expansion is the diversification of pollination and mating systems. The diversification is clearly reflected in the rapid and adaptive colonization of flowering plants under various environmental conditions (for review, see Holsinger and Steinbachs, 1997). Immobility of flowering plants requires biological structures (pollen) to deliver the male gametes to the enclosed female gametophyte by means of pollination. Diversification of pollination system manifests its selves in variations in pollen development (bi- and tricellular pollen), stigma development (wet and dry-type stigmas), pollination strategy (biotic and a-biotic pollinators), and in pollen recognition (self-compatibility and self-incompatibility). Diversification of the morphology of styles (closed, semi-closed and open styles), ovaries and of pollen tube attraction mechanisms is clearly a reflection of the broad variation in mating systems. Since transmission of genetic information to the next generation is mediated by reproductive systems, it is obvious that evolution of pollination and mating systems has led to evolutionary diversification and speciation. In this respect, a strong relationship exists between micro-evolutionary processes and macro-evolutionary patterns.

The term 'flowering plants' can be misleading, because not all angiosperm species have prominent flowers. However, all species do posses the kinds of reproductive structures like the pistil and stamen that are found within prominent flowers. These reproductive structures are responsible for the production of gametes (egg or sperm cells) within

gametophytes. The female gametophyte is a haploid multi-cellular structure called the embryo sac that develops within the ovule of the ovary. The ovary, style and stigma together represent the female reproductive organ or pistil (Fig. 1; for review, see Gasser and Robinson-Beers, 1993; Reiser and Fischer, 1993). The female gametophyte that consists of eight nuclei originates from a haploid megaspore that undergoes several haploid mitotic divisions in a process called megagametogenesis. The multi-cellular embryo sac is formed after compartmentalization of the nuclei in seven cells. Prior to fertilization, a fusion occurs between the two polar nuclei in the central cell. After release of two sperm cells from the male gametophyte into one of the synergid cells of the embryo sac, a double fusion process occurs of one sperm cell with the egg cell and of the other sperm cell with the central cell (containing the fused polar nuclei). Fusion of the sperm and egg cell leads to zygote formation, whereas fusion of the sperm and central cell gives rise to triploid endosperm. Division of the zygote finally gives rise to the formation and development of the embryo that is nourished by nutrient sources from the endosperm.

Pollen development and pollen tube growth

Despite the inconceivable variety in pollination and mating systems, reproductive processes are conserved among flowering plants. For example, the development of pollen within the anther is characterized by successive cytological and cytochemical alterations that have a high degree of similarity in both mono- and dicotyledonous plant species (for review, see Scott et al., 1991; Goldberg et al., 1993; McCormick, 1993). Pollen development starts with the formation of pollen mother cells in a process named microsporogenesis (Fig. 1). Enzymes and nutrients that are essential for microsporogenesis are provided by the tapetum, which is a glandular tissue that surrounds the pollen mother cells. Pollen mother cells are transformed into microspores by two meiotic divisions. After meiosis II, tetrads of microspores are formed through deposition of several layers of callose (1,3-β-glucan). During subsequent microspore development, secretion of callase by the tapetum degrades the adhesive callose layer that finally results in the release of individual microspores. The free microspores increase in volume as the result of the formation of endogenous small vacuoles that fuse to form one large vacuole. During degradation of the tapetum, a microspore undergoes an unequal or asymmetric mitotic division, named pollen mitosis I.

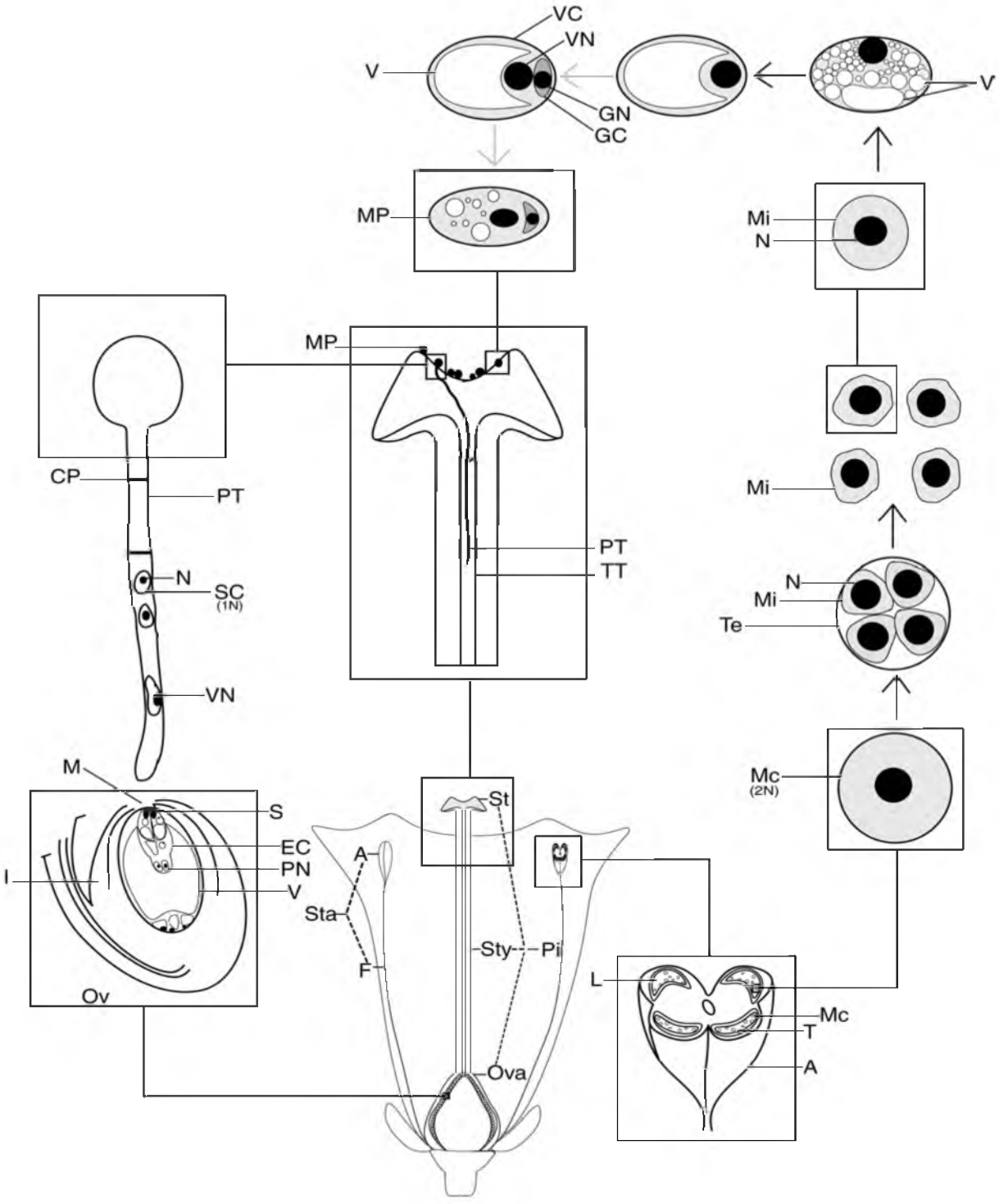

Figure 1. Schematic representation of male gametogenesis and pollen tube growth.

Development events related to microsporogenesis or microgametogenesis are indicated with black or gray arrows, respectively. Key for abbreviations: A, anther; CP, callose plug; EG, egg cell; F, filament; GC, generative cell; GN, generative nucleus; I, integument; L, locule; M, micropyle; Mc, microsporocyte; Mi, microspore; MP, mature pollen; N, nucleus; Ov, ovule; Ova, ovary; Pi, pistil; PN, polar nuclei; PT, pollen tube; S, synergid; St, stigma; Sta, stamen; Sty, style; SC, sperm cell; T, tapetum; Te, tetrad; TT, transmitting tract; V, vacuole; VC, vegetative cell; VN, vegetative nucleus.

From this division event on, the microspore is called pollen grain and its preceding development is named microgametogenesis. After pollen mitosis I, a pollen grain is a bicellular structure which consists of a small generative cell and a large vegetative cell. The generative cell usually undergoes a second mitotic division (pollen mitosis II) during pollen tube growth that results in the formation of two sperm cells. In species from the mustard (Crucifereae) and grass (Gramineae) families, however, formation of the two sperm cells is already established during pollen maturation (Brewbaker, 1967). Maturation of the pollen grain involves several processes and leads to a complete preparation of the grain for its final release from the anther (anthesis). The most noticeable process during the maturation of the pollen grain is its extensive dehydration (up to 60 %). This reversible dehydrated state allows the survival of the pollen grain under unfavorable environmental conditions (Lin and Dickinson, 1984). Comparable dehydration processes occur during seed and spore development (for review, see Ingram and Bartels, 1996).

A mature pollen grain is transported as a free structure by wind, water, animals or insects to the stigma of a flower. Pollination starts when a pollen grain lands on the surface of a compatible stigma (receptive surface; Fig. 1), which can be covered by an adhesive coating (dry-type stigma) or a liquid exudate (wet-type stigma; Heslop-Harrison and Shivanna, 1977). Although differences exist in the composition of the receptive surfaces between plant species, the main functions are protection of the pistil against pathogens and binding, recognition, rehydration, -and nutrition of pollen (for review, see De Graaf et al., 2001). Rehydration of the pollen grain on the stigma leads to a bulging out of the vegetative cell from a specific site in the pollen cell wall (the aperture) and the subsequent growth of a pollen tube into the stigma surface. Although pollen of different plant species can be urged to in vitro germination and tube growth using a sucrose and boric acid-rich medium (Read et al., 1993a, 1993b), little is known about the mechanisms underlying in vivo pollen tube growth. Despite differences between in vivo and in vitro growing pollen tubes (Kandasamy et al., 1992), the study of in vitro and in vivo pollen tube cultures is a first step towards clarification of processes involved in in vivo pollen tube growth (for review, see Derksen et al., 1995; Cheung, 1996; Malhó, 1998; De Graaf et al., 2001). Pollen tube growth is driven by an active distribution of different cell components towards the tip by means of cytoplasmic streaming. The cytoplasm contains the vegetative nucleus, the generative or sperm cells (after mitosis II), organelles and secretory vesicles. Fusion of secretory vesicles, containing cell wall precursor components, with the pollen tube tip membrane causes membrane enlargement and cell wall formation that leads to unidirectional pollen tube tip growth. During pollen tube growth, the cytoplasm

becomes separated from the rest of the pollen tube by a regular deposition of callose plugs along the whole length of the pollen tube (Fig. 1). After penetrating the stigma, pollen tubes grow through the intercellular space of the stylar transmitting tract, which is an extracellular matrix rich in polysaccharides, glycoproteins and glycolipids. As soon as a pollen tube penetrates the ovule via the micropyle, the tube bursts and the sperm cells are released into one of the synergids to fuse with the egg and central cell, respectively. From this moment on, fertilization is a fact and subsequent divisions of the zygote give rise to the development of the embryo and the genesis of a new individual.

In summary, pollen development and pollen tube growth are fascinating and unique phenomena to study, because they constitute several physiologically and biochemically related processes that occur in a relatively short period. Moreover, as soon as a mature pollen grain lands on a compatible stigma, development programs of both male and female gametophytes are initiated immediately and cooperatively in order to direct a successful delivery of the male gamete into the ovule. Although various differences exist in the morphology and physiology of flower organs between plant species, it is apparent that these development programs exhibit a high level of conservation.

Transcription during microgametogenesis: origin, fate and uniqueness of pollen mRNA

The previous section clearly illustrates that the male gametophyte undergoes complex developmental programs to reach maturity. It is apparent that these programs are conserved between plant species throughout evolution. In this respect, it is obvious that the complexity and extent of conservation of the developmental programs are reflected in the organization of genetic programs that determine gene activity in pollen. Gene activity in pollen comprises the transcription of at least 20,000 genes in spiderwort (Willing and Mascarenhas, 1984) and 24,000 different genes in maize (Willing et al., 1988). Due to gene redundancy, it is thinkable that the exact number of transcribed genes in pollen is even higher. In both mono- and dicotyledonous plant species, a considerable part of the total amount of mRNA originates from a relative small number of genes with a high mRNA copy number. In spiderwort pollen, for example, transcriptional activity of approximately 40 genes covers about 15 % of the total mRNA population. In maize, 35 % of the total mRNA population is synthesized by only 240 genes. Genetic studies based on dimerization properties of isozymes (multimeric enzymes) indicate that synthesis of microspore and pollen mRNAs starts after meiosis II, i.e. from a

haploid genome (Weeden and Gottlieb, 1979; Tanksley et al., 1981; Singh et al., 1985; Sari-Gorla et al., 1986; Pedersen et al., 1987). Although the genetic studies indicate that microspore and pollen mRNAs are synthesized solely after meiosis, pre-meiotic origin cannot be excluded yet. Recent progress in the isolation and characterization of microsporogenic cDNAs (F. Cnudde, unpublished data) should enable to gain more insight in the degree of pre- and post-meiotic transcription during male gametogenesis.

With regard to the fate of mRNAs during microspore and pollen development from different plant species, much insight has been obtained from Northern analysis. Based on the hybridization patterns of a small number of genomic and cDNA clones, mRNA accumulation patterns have initially been divided into two populations; those of the early and those of the late transcripts (Stinson et al., 1987). The early transcripts have their maximum accumulation level in microspores, whereas the late transcripts accumulate to a maximum level after pollen mitosis I. Comparable mRNA accumulation patterns have been found for pollen of tobacco and lily by means of in vitro translation experiments (Schrauwen et al., 1990). Additional and more substantial evidence for the presence of various mRNA populations in developing microspores and pollen from different plant species is presented in Figure 2A. A literature study to mRNA accumulation patterns of pollen-expressed genes from different plant species revealed the presence of at least seven different mRNA accumulation patterns or clusters that can be categorized into four main populations (Fig. 2A). Population 1 consists of mRNAs that show their highest accumulation level in the developing microspore, where transcripts of population 2 increase in their accumulation level during pollen development with a maximum in mature pollen. Transcripts of population 3 exhibit a maximum accumulation level in immature bi- or tricellular pollen. Finally, mRNAs from population 4 are characterized by their low and constitutive accumulation level throughout pollen development. As illustrated in Figure 2B, a large number of the mRNAs from population 2 (for example *ntp303* and *rop1at*) shows a continuation of the high transcript accumulation level during subsequent pollen tube growth. Besides the continuing course, the transcript accumulation levels of a small number of mRNA species of populations 2 (for example *sb401* and *nsaap1*) and 4 (*zmmads2* and *sbpk*) or populations 2 (*tpc70*) and 3 (*pgps/d8* and *pgps/nh21*) respectively increase or decrease at the onset of pollen tube growth. The results of both the in vitro translation experiments and the Northern analyses clearly indicate that the fate of pollen transcripts from different plant species is reflected in the presence of several mRNA populations that are distinguishable in their accumulation kinetics.

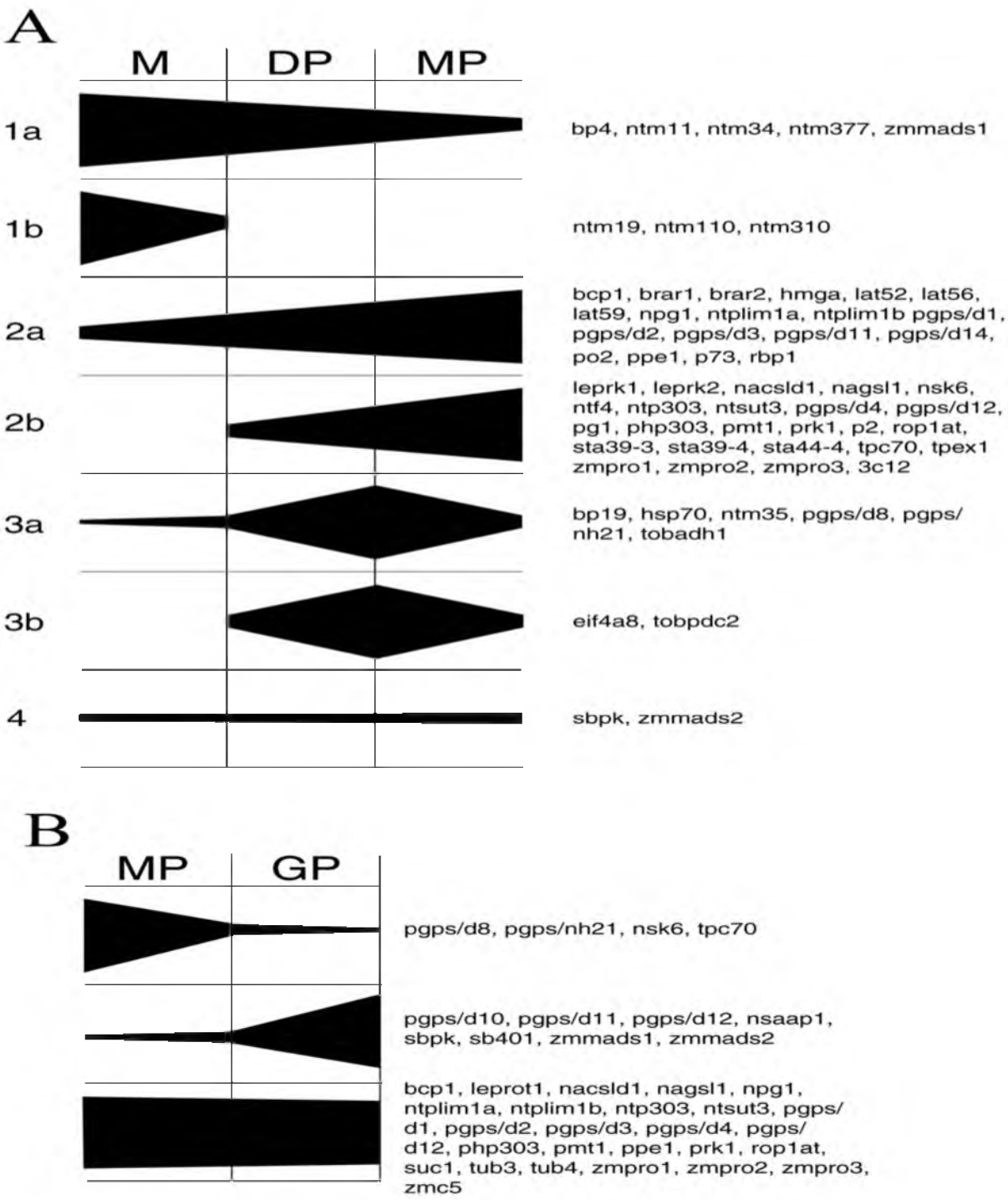

Figure 2. Schematic representation of mRNA accumulation patterns of pollen-expressed genes.

Transcript accumulation patterns in developing pollen (A) or in mature pollen and pollen tubes (B). The mRNA accumulation patterns reflect the course of accumulation of individual mRNAs as determined by Northern blot analysis, i.e. the tapering of each accumulation pattern reflects the decrease or increase of the mRNA accumulation level. Key for abbreviations: M, microspore; DP, developing pollen; MP, mature pollen; GP, germinating pollen. For references of the genes, see Table I. For reference "ntm"-clones, see Oldenhof et al. (1997).

To what extent are pollen-expressed genes active in non-gametophytic tissues? Data obtained from electrophoretic studies of various isozymes revealed an extensive overlap of gametophytic and sporophytic gene expression. In barley, 60 % of 50 different isozymes were found to be present in both gametophytic and sporophytic tissues (Pedersen et al., 1987). A similar percentage of gametophytic-sporophytic overlap was found for tomato (Tanksley et al., 1981), whereas 34 isozyme profiles in maize revealed an overlap of 72 % (Sari-Gorla et al., 1986). Extensive overlap of gametophytic and sporophytic gene expression in both mono- and dicotyledonous plants has been confirmed by means of cDNA-mRNA hybridization experiments. For example, at least 64 % of the mRNAs in pollen of spiderwort and maize was also found to be present in shoot tissue (Willing and Mascarenhas, 1984; Willing et al., 1988). Besides the gametophytic and sporophytic overlap of expression of a large number of genes, the hybridization experiments indicated also the presence of "pollen-specific" gene activity (Stinson et al., 1987). However, recent progress in the understanding of plant gene expression argues more and more against the presence of unique mRNAs in specific cell types like pollen. For example, from the large number of pollen-expressed genes that has been isolated in the last 20 years (Table I), only a very small group appears to be exclusively expressed in pollen (Brown and Crouch, 1990; Reijnen et al., 1991; Weterings et al., 1992; Tebbutt et al., 1994; Zou et al., 1994; Rubinstein et al., 1995a, 1995b; Christensen et al., 1996; Liu et al., 1997; Muschietti et al., 1998; Stratford et al., 2001). Nevertheless, it can be assumed that many of these "pollen-specific" genes are also expressed in sporophytic tissues. Since the expression of the "pollen-specific" genes has not been investigated in a large number of different sporophytic tissues of different developmental phases and under various environmental conditions, expression sites can easily have been missed. Sporophytic expression of "pollen-specific"' genes is also likely in view of the fact that many "pollen-specific" genes encode for products that may not necessarily perform a pollen-unique function (Table I). Examples of these products are profilin (*pfn4*; Christensen et al., 1996), receptor proteins (*leprk1*, *leprk2*; Muschietti et al., 1998), extensins (*mpex1*; Rubinstein et al., 1995a, 1995b; Stratford et al., 2001, *mpex2*, *tpex*; Stratford et al., 2001), polygalacturonases (*p2*; Brown and Crouch, 1990, *npg1*; Tebbutt et al., 1994), and receptor-like protein kinase (*prk1*; Mu et al., 1994a).

It is obvious that the actual pollen transcriptome is much more complex than outlined here and that differences in mRNA accumulation kinetics remain between different plant species. However, the overall presence of similar development-related mRNA populations in

Species	Clone	Homology	Reference
Arabidopsis thaliana	askβ	GSK-3/shaggy-like protein kinase	Tichtinsky et al. (1998)
Arabidopsis thaliana	ata18	-	Rubinelli et al. (1998)
Arabidopsis thaliana	ata21	-	Rubinelli et al. (1998)
Arabidopsis thaliana	atbnh	-	Leprince et al. (1999)*
Arabidopsis thaliana	atrab2	rab protein	Moore et al. (1997)
Arabidopsis thaliana	at59 (a2)	pectate lyase-like protein	Kulikauskas and McCormick (1997)
Arabidopsis thaliana	gmd1	GDP-D-mannose 4,6-dehydratase	Bonin and Reiter (1999)*
Arabidopsis thaliana	lpd2	lipoamide dehydrogenase	Lutziger and Oliver (2000)
Arabidopsis thaliana	pab5	poly(A)-binding protein	Belostotsky and Meagher (1993)
Arabidopsis thaliana	pfn4	profilin	Christensen et al. (1996)
Arabidopsis thaliana	prf4	profilin	Huang et al. (1996)
Arabidopsis thaliana	rac2	rac protein	Kost et al. (1998)*
Arabidopsis thaliana	rkf1	receptor-like kinase	Takahashi et al. (1998)
Arabidopsis thaliana	rop1at	rho-related GTPase	Li et al. (1998)
Arabidopsis thaliana	rop4	rho-like GTP-binding protein	Li et al. (1998)
Arabidopsis thaliana	rop6	rho-like GTP-binding protein	Li et al. (1998)
Arabidopsis thaliana	suc1	sucrose-H^+ transporter protein	Sauer and Stolz (1994)
Arabidopsis thaliana	tua1	α-tubulin	Ludwig et al. (1988)
Brassica napus	bp4a	-	Albani et al. (1990)
Brassica napus	bp4c	-	Albani et al. (1990)
Brassica napus	bp19	pectin esterase	Albani et al. (1991)
Brassica napus	hmga	HMG I/Y-like protein	Masek et al. (2000)
Brassica napus	rbp1	RNA-binding protein	Smýkal et al. (2000)
Brassica napus	sta39-3	arabinogalactan protein	Gerster et al. (1996)
Brassica napus	sta39-4	arabinogalactan protein	Gerster et al. (1996)
Brassica napus	sta44-4	polygalacturonase	Robert et al. (1993)
Brassica rapa	bcp1	-	Theerakulpisut et al. (1991) Xu et al. (1995)
Brassica rapa	brar1	Ca^{2+}-binding protein	Toriyama et al. (1995)
Brassica rapa	brar2	Ca^{2+}-binding protein	Toriyama et al. (1995)
Helianthus annuus	plim1a (sf3)	LIM domain protein	Baltz et al. (1992)
Helianthus annuus	plim2	LIM domain protein	Baltz and Steinmetz (1998)*
Helianthus annuus	sf16	-	Dudareva et al. (1994)
Helianthus annuus	sf17	-	Reddy et al. (1994)*
Hordeum bulbosum	trx	thioredoxin-like protein	Juttner et al. (1999)*
Ipomoea trifida	isp11	-	Kowyama et al. (1995)*
Lilium longiflorum	a23	-	Chang and Wang (1999)
Lilium longiflorum	ltp	lipid transfer protein	Park et al. (1999)*
Lilium longiflorum	L18909**	thioredoxin-like protein	Kim et al. (1993)*
Lilium longiflorum	L18911**	pectate lyase-like	Kim et al. (1993)*
Lilium longiflorum	p35	-	Tanaka et al. (1999)
Lilium longiflorum	y5-7	actin-bundling protein	Vidali et al. (1999)
Lilium longiflorum	Z17328**	pectate lyase-like	Kim et al. (1992)*
Lilium longiflorum	14-3-3	14-3-3-like protein	Pertl et al. (1999)*
Lolium perenne	trx	thioredoxin-like protein	Juttner et al. (1999)*
Lycopersicon esculentum	lat52	-	Twell et al. (1989b)
Lycopersicon esculentum	lat56	pectate lyase	Twell et al. (1991) Wing et al. (1989)
Lycopersicon esculentum	lat59	pectate lyase	Twell et al. (1991) Wing et al. (1989)
Lycopersicon esculentum	leprk1	receptor-like protein kinase	Muschietti et al. (1998)
Lycopersicon esculentum	leprk2	receptor-like protein kinase	Muschietti et al. (1998)
Lycopersicon esculentum	leprot1	proline transporter protein	Schwacke et al. (1999)
Lycopersicon esculentum	leprot2	proline transporter protein	Schwacke et al. (1999)
Lycopersicon esculentum	tpex (pex1)	pollen extensin-like protein	Stratford et al. (2001)
Medicago sativa	po2	arabinogalactan-like protein	Qiu et al. (1997)
Medicago sativa	p73	polygalacturonase	Qiu and Erickson (1996)
Nicotiana alata	nacsld1	β-glucan synthase	Doblin et al. (2001)
Nicotiana alata	nagsl1	β-glucan synthase	Doblin et al. (2001)
Nicotiana sylvestris	nsaap1	amino acid permease	Lalanne et al. (1995) Lalanne et al. (1997)
Nicotiana tabacum	eif4a8	RNA helicase	Brander and Kuhlemeier (1995)
Nicotiana tabacum	jd1	-	Nardi et al. (2000)*
Nicotiana tabacum	npg1 (tp27)	polygalacturonase	Tebbutt et al. (1994)
Nicotiana tabacum	nsk6	shaggy-like kinase protein kinase	Takvorian et al. (1996)*
Nicotiana tabacum	nsk59	GSK-3/shaggy-like protein kinase	Tichtinsky et al. (1998)
Nicotiana tabacum	nsk91	GSK-3/shaggy-like protein kinase	Tichtinsky et al. (1998)
Nicotiana tabacum	nsk111	GSK-3/shaggy-like protein kinase	Tichtinsky et al. (1998)
Nicotiana tabacum	ntf4	MAP kinase	Wilson et al. (1995)
Nicotiana tabacum	nthsp18p	heat shock protein	Žárský et al. (1995)
Nicotiana tabacum	ntplim1a	LIM domain protein	Sweetman et al. (2000)

Nicotiana tabacum	ntplim1b	LIM domain protein	Sweetman et al. (2000)
Nicotiana tabacum	ntpro2	profilin	Mittermann et al. (1996)
Nicotiana tabacum	ntpro3	profilin	Mittermann et al. (1996)
Nicotiana tabacum	ntp303	-	Weterings et al. (1992, 1995a)
Nicotiana tabacum	ntsut3	sucrose transporter-like protein	Lemoine et al. (1999)
Nicotiana tabacum	nt59	pectate lyase-like protein	Kulikauskas and McCormick (1997)
Nicotiana tabacum	plim2	LIM domain protein	Eliasson et al. (1998)*
Nicotiana tabacum	pronp1	profilin	Swoboda et al. (2001)
Nicotiana tabacum	p18	-	Mol (1998)*
Nicotiana tabacum	rop1	GTPase	Cvrckova and Žárský (1999)
Nicotiana tabacum	tac9	actin	Thangavelu et al. (1993)
Nicotiana tabacum	tac25	actin	Thangavelu et al. (1993)
Nicotiana tabacum	tobadh1	alcohol dehydrogenase	Op den Camp and Kuhlemeier (1997)
Nicotiana tabacum	tobpdc2	pyruvate decarboxylase	Bucher et al. (1995)
Nicotiana tabacum	tp5	β-galactosidase-like	Maund and Johnson (1999)*
Nicotiana tabacum	tp10 (g10)	pectate lyase-like protein	Rogers et al. (1992)
Nicotiana tabacum	136.1	membrane integral-like protein	Rodin et al. (1995)*
Oenothera organensis	p2	polygalacturonase	Brown and Crouch (1990)
Oryza sativa	ps1	-	Zou et al. (1994)
Petunia hybrida	pgps/d1	LIM domain protein	Guyon et al. (2000)
Petunia hybrida	pgps/d2	-	Guyon et al. (2000)
Petunia hybrida	pgps/d3	neuromodulin-like protein	Guyon et al. (2000)
Petunia hybrida	pgps/d4	polygalacturonase-inhibiting protein	Guyon et al. (2000)
Petunia hybrida	pgps/d6	ER lumen protein receptor	Guyon et al. (2000)
Petunia hybrida	pgps/d8	cytochrome c oxidase	Guyon et al. (2000)
Petunia hybrida	pgps/d10	-	Guyon et al. (2000)
Petunia hybrida	pgps/d11	arabinogalactan-like protein	Guyon et al. (2000)
Petunia hybrida	pgps/d12	-	Guyon et al. (2000)
Petunia hybrida	pgps/d14	-	Guyon et al. (2000)
Petunia hybrida	pgps/nh21	Zn metallothionein	Guyon et al. (2000)
Petunia hybrida	pmt1	monosaccharide transporter protein	Ylstra et al. (1998)
Petunia hybrida	php303 (p303)	-	Hulzink et al. (2002)***
Petunia inflata	ppe1 (pepe22)	pectin esterase	Mu et al. (1994b)
Petunia inflata	prk1	receptor-like kinase	Mu et al. (1994a)
Pyrus pyrifolia	AB013353**	UDP-glucose pyrophosphorylase	Kiyozumi et al. (1999)
Solanum berthaultii	sbpk	protein kinase	Liu et al. (1999)
Solanum berthaultii	sb401	-	Liu et al. (1997)
Solanum chacoense	AF161330**	-	Luu et al. (1999)*
Solanum tuberosum	invge	invertase	Maddison et al. (1999)
Solanum tuberosum	invgf	invertase	Maddison et al. (1999)
Tradescantia paludosa	tpc70	-	Turcich et al. (1994)
Zea mays	hsp70	heat shock protein	Gagliardi et al. (1995)
Zea mays	mpex1	pollen extensin-like protein	Rubinstein et al. (1995a) Stratford et al. (2001)
Zea mays	mpex2	pollen extensin-like protein	Stratford et al. (2001)
Zea mays	pg1	polygalacturonase	Niogret et al. (1991)
Zea mays	tua1 (mg19/6)	α-tubulin	Montoliu et al. (1989) Rigau et al. (1993)
Zea mays	tua4	α-tubulin	Dolfini et al. (1993) Villemur et al. (1992)
Zea mays	tua6	α-tubulin	Villemur et al. (1992)
Zea mays	tub3	β-tubulin	Rogers et al. (1993)
Zea mays	tub4	β-tubulin	Rogers et al. (1993)
Zea mays	tub5	β-tubulin	Rogers et al. (1993)
Zea mays	zmabp1	actin-depolymerizing factor	Rozycka et al. (1995)
Zea mays	zmc5	pectin methylesterase-like protein	Wakeley et al. (1998)
Zea mays	zmmads2	MADS box transcription factor	Heuer et al. (2000)
Zea mays	zmmads1	MADS box transcription factor	Heuer et al. (2001)
Zea mays	zmpro1	profilin	Staiger et al. (1993)
Zea mays	zmpro2	profilin	Staiger et al. (1993)
Zea mays	zmpro3	profilin	Staiger et al. (1993)
Zea mays	zm13	-	Hamilton et al. (1989) Hanson et al. (1989)
Zea mays	3c12	polygalacturonase	Allen and Lonsdale (1992) Allen and Lonsdale (1993) Rogers et al. (1991)

Table I. Genes expressed in developing or germinating pollen.

Updated list of genes (cDNA and genomic clones) that are highly expressed in pollen or pollen tubes. Genes encoding for pollen coat proteins and pollen allergens are excluded. * indicates unpublished (reference is derived from the NCBI GenBank database). ** indicates GenBank accession number (in case of absence of a clone name). *** means published in the present thesis.

microspores, pollen grains and pollen tubes in different plant species argues clearly for conservation of genetic programs in the male gametophyte throughout evolution.

Translation during pollen development and pollen tube growth: one plus one is not always two

Translation in eukaryotic cells is a very complex process and is regulated at many levels. The most basal level is the availability of various macromolecules: translation factors (initiation factors, elongation factors, and termination factors), RNA-binding proteins, ribosomes (ribosomal proteins), ribosomal RNA (rRNA), transfer RNA (tRNA), and mRNA (for review, see Kozak, 1992; Browning, 1996; El'-skaya, 1999; Kozak, 1999). For pollen, few data are available concerning the expression of genes that encode for translation factors or RNA-binding proteins (Owttrim et al., 1991; Belostotsky and Meagher, 1993; Brander and Kuhlemeier, 1995; Belostotsky and Meagher, 1996; Smýkal et al., 2000). In contrast to the availability of translation factors or RNA-binding proteins, much more is known about the presence of rRNA, tRNA and ribosomes in pollen from different plant species. Research on rRNA and tRNA synthesis in pollen was initiated in the late sixties and comprised the semi-quantitative analysis of ^{32}P-labeled inorganic phosphate incorporation in RNA molecules (for review, see Mascarenhas, 1975, 1990). These so-called "feed and chase" experiments showed a high level of rRNA synthesis in microspores of lily and spiderwort, which was absent during pollen tube growth. Similar synthesis patterns were found for tRNA and ribosomes. However, rRNA and tRNA synthesis persists during germination of *Nicotiana alata* pollen (Tupý et al., 1977; Süss and Tupý, 1978), emphasizing the existence of differences in the kinetics of rRNA and tRNA synthesis between plant species. Regardless of their synthesis rate, accumulation of rRNA, tRNA and ribosomes persists throughout pollen development and pollen tube growth in various plant species (Mascarenhas, 1975; Tupý, 1977; Hoekstra and Bruinsma, 1979).

As described in the previous section, mature pollen grains and pollen tubes contain a large stock of mRNA species that are represented in different accumulation populations. In order to investigate to what extent these mRNAs are translated during pollen development and pollen tube growth, one- and two-dimensional electrophoresis experiments of pollen and pollen tube proteins have been carried out (Mascarenhas, 1975; Žárský et al., 1985; Čapková et al., 1987; Van Herpen et al., 1992; Mascarenhas, 1993). Protein patterns from mature

pollen and pollen tubes exhibited a high qualitative similarity, indicating that many proteins persist during the transition of developing to germinating pollen. However, several studies have demonstrated the importance of newly synthesized proteins for pollen tube growth (Čapková et al., 1987, 1988, 1994; Mascarenhas, 1993). Transcription inhibition experiments showed that protein synthesis during pollen tube growth occurs mainly independent of new mRNA synthesis (Čapková et al., 1988; Mascarenhas, 1993; Štorchová et al., 1994). Thus, protein synthesis at the onset of pollen tube growth uses pre-synthesized mRNAs that are stored in mature pollen. The presence of stored mRNA has been demonstrated in pollen grains of spiderwort (Frankis and Mascarenhas, 1980), maize (Mascarenhas et al., 1984), tobacco (Schrauwen et al., 1990; Štorchová et al., 1994), and lily (Schrauwen et al., 1990). These observations clearly indicate that post-transcriptional control of gene expression is very important for pollen tube growth in various plant species. Despite rapid progress in cloning and characterization of pollen-expressed genes, only a few examples exist in literature describing delayed translation of pollen mRNAs. A well-documented example is *ntp303* from tobacco. Recent studies have provided several lines of evidence that demonstrate the post-transcriptional regulation of NTP303 synthesis. One obvious clue is the discrepancy between the accumulation patterns of *ntp303* mRNA and NTP303 protein during pollen development. Transcripts of the *ntp303* gene are first detectable after pollen mitosis I and continue to accumulate during pollen maturation and pollen tube growth (Weterings et al., 1992). Despite the abundant accumulation of the mRNA during pollen development, Western blot analysis revealed the appearance of the NTP303 protein only at the onset of pollen germination (Wittink et al., 2000). These observations clearly indicate lack of NTP303 protein synthesis during pollen development, which is followed by an enhancement of its synthesis during pollen germination and pollen tube growth.

Despite the general occurrence of post-transcriptional regulation of protein synthesis in pollen from various plant species, regulation of translation is still a rather unexplored field within plant research. The lack of knowledge is rather striking, since pollen represent an almost ideal model system to investigate basal and specific principles of mRNA translation. Development of pollen and growth of pollen tubes can easily be performed on a large scale in in vitro cultures (Tupý et al., 1991; Van Herpen et al., 1992; Read et al., 1993a, 1993b), which enables study of translation in a population of homogeneous cells under controlled environmental conditions. Furthermore, several features of translation in pollen of both mono- and dicotyledonous plant species, like storage of different components of the translation machinery and enhanced translation of transcripts, are also found in developing gametes from

invertebrates and mammals (Schäfer et al., 1995; Sommerville and Ladomery, 1996; Steger, 2001). The existence of these parallels might indicate conservation of translation regulatory mechanisms between animals and plants and could thus provide unique possibilities for comparative studies.

Regulation of pollen gene expression: the role of regulatory sequences

The extent of conservation of genetic programs in pollen from different plant species becomes even more apparent at the molecular level. The promoter of many pollen-expressed genes that encode for a variety of products and that originate from different plant species contains several sequence elements that are highly conserved (Table II). Promoter research of pollen-expressed genes has been initiated by Twell and coworkers in the late eighties. Their first set of experiments involved the analysis of transient expression of *gus* reporter gene constructs containing the promoter of a pollen-expressed gene (*lat52*) after introduction into tobacco pollen and leaves by means of particle bombardment (Twell et al., 1989a). The successful application of particle bombardment for the delivery of reporter gene constructs in pollen has led to similar promoter analyses of pollen-expressed genes from various plant species (for example, Nishihara et al., 1993; Twell et al., 1993; Zou et al., 1994; Lonsdale et al., 1995; Keller and Hamilton, 1998; Gerola et al., 2000; Hamilton et al., 2000; Stratford et al., 2001). Promoter studies of pollen-expressed genes were also performed using stable transformants (for example, Van Tunen et al., 1990; Carpenter et al., 1992; Allen and Lonsdale, 1993; Twell et al., 1993; Xu et al., 1993; Eady et al., 1994; Zou et al., 1994; Brander and Kuhlemeier, 1995; Custers et al., 1997; Moore et al., 1997; Li et al., 1998; Hamilton et al., 2000; Drea et al., 2001).

Profound functional promoter analyses have been performed for the pollen-expressed genes *g10* (tobacco; Rogers et al., 2001), *lat52* (tomato; Twell et al., 1991; Eyal et al., 1995; Bate and Twell, 1998), *lat59* (tomato; Twell et al., 1991; Eyal et al., 1995), *npg1* (tobacco; Tebbutt and Lonsdale, 1995), *ntp303* (tobacco; Weterings et al., 1995b), and *zm13* (maize; Guerrero et al., 1990; Hamilton et al., 1992, 1998). The *lat52* promoter contains three regions (A, B and C) that exhibit a distinct effect on stable expression upon deletion (Twell et al., 1991). The proximal region (region C) determines a basal expression level in pollen, whereas the upstream regions A and B are required for a high level of gene expression. Promoter gain-of-function analysis revealed that *lat52* expression is determined by a differential activation of

the three promoter regions during pollen development (Eyal et al., 1995; Bate and Twell, 1998). The regions A and B mediate a high level of gene expression specifically during early pollen development (early to mid-bicellular pollen), whereas the activity of all three regions enables a high level of expression in mid- and late-bicellular pollen. Like region C of *lat52*, the proximal promoter region of several other pollen-expressed genes that originate from both mono- and dicotyledonous plant species are important for pollen-preferential expression. Examples of such genes are *g10* (Rogers et al., 2001) *lat59* (Twell et al., 1991; Eyal et al., 1995), *lat56* (Twell et al., 1991), *npg1* (Tebbutt and Lonsdale, 1995), *ntp303* (Weterings et al., 1995b), *slg* (Dzelzkalns et al., 1993), *tua1* (Carpenter et al., 1992), and *zm13* (Hamilton et al., 1992, 1998). Besides the proximal region, several other functional promoter regions have been shown to alter pollen gene expression either positively (Twell et al., 1991; Carpenter et al., 1992; Dzelzkalns et al., 1993; Xu et al., 1993; Tebbutt and Lonsdale, 1995; Weterings et al., 1995b; Mitsuda et al., 2001; Rogers et al., 2001) or negatively (Twell et al., 1991; Hamilton et al., 1992; Xu et al., 1993; Tebbutt and Lonsdale, 1995). In several cases, the functional regions act in a co-dependent manner (like the Q-element in *zm13*; Hamilton et al., 1998 and element I and III in *S_2-RNase*; Ficker et al., 1998), whereas in other cases the regions are able to function more or less independently (like part of the C region in *lat52*; Eyal et al., 1995; Bate and Twell, 1998).

Alignment of promoter sequences of three similar expressed pollen genes from tomato, *lat52* (Twell et al., 1989b), *lat56* and *lat59* (Wing et al., 1989), revealed the presence of several conserved elements, like the 52/56 element, the PB core element and the 56/59 element (Table II; Twell et al., 1991). The 52/56 element is a 12 base pairs sequence (TGTGGTTATATA) which is completely conserved between the *lat52* and *lat56* promoters. Variants of the 52/56 element have been found, among others, in the promoter of the pollen-expressed gene *eif4a8* (Brander and Kuhlemeier, 1995), *g10* (Rogers et al., 2001), *npg1* (Tebbutt and Lonsdale, 1995), and *ntp303* (Weterings et al., 1995a). The 52/56 element of the *lat52* promoter is essential for directing a high level of expression in pollen (Twell et al., 1991). This has been confirmed for a 52/56 element variant in the promoter of the pollen-expressed gene *npg1* from tobacco (Tebbutt and Lonsdale, 1995).

A stretch of seven base pairs in the 52/56 element, named the PB core element (TGTGGTT), is repeated three times in the promoter of *lat52* (PB-I - III). A series of scanning mutations through the 52/56 element (including the PB-I element) of the *lat52* promoter revealed the requirement of the GTGG residues for normal gene activity in pollen, whereas two adjoining TTAT elements negatively influenced gene expression (Bate and

Element	Gene	Reference	Element	Gene	Reference
52 /56 element				mudr	Raizada et al. (2001)
TGTGGTTATATA	eif4a8	Brander and Kuhlemeier (1995)		npg1	Tebbutt and Lonsdale (1995)
	lat52	Twell et al. (1991)		ntp303	Wetering et al. (1995a, b)
	lat56	Twell et al. (1991)		nt59	Kulikauskas and McCormick (1997)
	npg1	Tebbutt and Lonsdale (1995)		pi	Lonsdale et al. (1995)
	ntp303	Wetering et al. (1995 a, b)		ps1	Zou et al. (1994)
	psst	Heiser et al. (1996)		rop1at	Li et al. (1998)
	tp10	Rogers et al. (2001)		tpex	Stratford et al. (2001)
	tyky	Zabaleta et al. (1998)		tua1	Carpenter et al. (1992)
	55kDa	Zabaleta et al. (1998)		3c12	Allen and Lonsdale (1993)
PB core element			**AAATGA element**		
TGTGGTT	atrab2	Moore et al. (1997)	AAATGA	atrab2	Moore et al. (1997)
	eif4a8	Brander and Kuhlemeier (1995)		bp19	Albani et al. (1992)
	invge	Maddison et al. (1999)			
	invgf	Maddison et al. (1999)		h^+-ppase	Mitsuda et al. (2001)
	lat52	Bate and Twell (1998) Twell et al. (1991)		invgf	Maddison et al. (1999)
	lat56	Bate and Twell (1998) Twell et al. (1991)		nsaap1	Lalanne et al. (1997)
	lat59	Bate and Twell (1998) Twell et al. (1991)		ntf4	Voronin et al. (2001)
	npg1	Tebbutt and Lonsdale (1995)		ntp303	Wetering et al. (1995a, b)
	nsaap1	Lalanne et al. (1997)		po2	Wu et al. (1998)
	ntp303	Wetering et al. (1995a, b)		po22	Wu et al. (1998)
	ps1	Zou et al. (1994)		po149	Wu et al. (1998)
	psst	Heiser et al. (1996)	**Q element**		
	tyky	Zabaleta et al. (1998)	AGGTCA	h^+-ppase	Mitsuda et al. (2001)
	tua1	Carpenter et al. (1992)		mudr	Raizada et al. (2001)
	zm13	Hamilton et al. (1989)		zm13	Hamilton et al. (1989)
	3c12	Allen and Lonsdale (1993)	**AGAAA element**		
	55kDa	Zabaleta et al. (1998)	AGAAA	invge	Maddison et al. (1999)
56 /59 element				invgf	Maddison et al. (1999)
GAATTTGTGA	ac	Lonsdale et al. (1995)		lat52	Bate and Twell (1998)
	atrab2	Moore et al. (1997)		lpd2	Drea et al. (2001)
	at59	Kulikauskas and McCormick (1997)	**TCCACCATA element**		
	eif4a8	Brander and Kuhlemeier (1995)	TCCACCATA	invge	Maddison et al. (1999)
	g10	Rogers et al. (2001)		invgf	Maddison et al. (1999)
	invge	Maddison et al. (1999)		lat52	Bate and Twell (1998)
	invgf	Maddison et al. (1999)		npg1	Tebbutt and Lonsdale (1995)
	lat56	Twell et al. (1991)		ps1	Zou et al. (1994)
	lat59	Twell et al. (1991)		ps1	Zou et al. (1994)

Table II. Conservation of pollen promoter elements in various genes.

Updated list of promoter elements that have been demonstrated to be of importance for gene expression in the male gametophytic.

Twell, 1998). In contrast to the 52/56 element, the PB core element is more frequently present in the promoter of pollen-expressed genes that originate from different plant species (Table II).

The 56/59 element is a 10 base pairs sequence (GAATTTGTGA) which is completely conserved between the *lat56* and *lat59* promoters. In both promoters, the 56/59 element is located at the same distance from the transcription initiation site and is thought to be of importance for basal expression in pollen (Twell et al., 1991). Mutation of the GTGA element in the promoter of *lat56* and *lat59* strongly reduced expression in pollen, which indicates the importance of these base pairs (Bate and Twell, 1998). This was confirmed by mutation of the GTGA sequence in the 56/59 element of the tobacco homologue of *lat56*, *g10* (Rogers et al., 2001). Furthermore, deletion of a variant of the 56/59 element (containing the GTGA core sequence) in the promoter of *npg1* led to a similar decrease in pollen gene expression (Tebbutt and Lonsdale, 1995).

Deletion and mutagenesis analyses have also demonstrated the presence of other promoter elements that exhibit a modulating effect on pollen gene expression. Examples are the *ntp303* AAATGA element (Weterings et al., 1995a, 1995b) and the *zm13* Q element (AGGTCA; Hamilton et al., 1998). Interestingly, the Q element and a variant of the 56/59 element (including the GTGA core sequence) are also present in the promoter region of the maize transposon *mudrB* gene (Raizada et al., 2001). The maize *mudrB* gene encodes for mutator (mu) transposon elements that are inserted at a high frequency in developing microspores. Fusion of a sequence region containing the *mudrB* promoter with the *gus* coding region revealed a 30-fold higher level of expression in mature maize pollen than in leaves, demonstrating the reason for the pollen-preferential mode of action of the transposon.

In conclusion, the overall presence of conserved promoter elements among genes that encode for different products and that originate from different plant species unmistakably argues for a functional and structural conservation of regulatory gene parts that are important for pollen gene expression throughout plant evolution. Although much progress has been made in the characterization of pollen gene promoters, conservation of several other promoter elements between pollen-expressed genes from various plant species (Van Tunen et al., 1989; Albani et al., 1991; Twell et al., 1991; Carpenter et al., 1992; Zou et al., 1994; Kulikauskas and McCormick, 1997; Hamilton et al., 1998; Maddison et al., 1999) argues for the presence of additional putative regulatory promoter elements that play a role in pollen mRNA synthesis.

Outline and aim of the thesis

In contrast to promoter research, much less data are available about the regulatory role of untranslated regions (UTRs) in directing gene expression in pollen (Dawe et al., 1993; Bate et al., 1996; Curie and McCormick, 1997). Both 5'- and 3'-UTRs of several genes have been shown to be of importance for alteration of gene expression in plants and animals (for review, see Gallie, 1993, 1996; Curtis et al., 1995; Fütterer and Hohn, 1996; Pain, 1996; Day and Tuite, 1998; Bailey-Serres, 1999). Translation of transcripts of many of these genes is often regulated at the initiation level by the 5'-UTR. In plants, the role of the 5'-UTR in the regulation of gene expression has been analyzed best for chloroplast-expressed genes (for review, see Danon, 1997). Several regulatory elements within the 5'-UTR of chloroplast-expressed genes have been identified and their interaction with RNA-binding proteins have been demonstrated (Dickey et al., 1998; Ling et al., 2000). The ability of the 5'-UTR to alter gene expression in pollen has been described only for a few genes, such as *lat59* (Curie and McCormick, 1997), *lat52* (Bate et al., 1996), and *adh1* (Dawe et al., 1993). Deletion analysis of the *lat59* 5'-UTR revealed the presence of two functional regions that account for inhibition of gene expression in pollen at the transcriptional level (Curie and McCormick, 1997). Mutation analysis of one of the 5'-UTR regions indicated that structural characteristics rather than the primary sequence determine the inhibitory activity. 5'-UTR-mediated enhancement of gene expression in pollen has been demonstrated for the *lat52* gene (Bate et al., 1996). Transient and stable expression analyses showed the capacity of the *lat52* 5'-UTR to enhance reporter gene expression in developing pollen at the post-transcriptional level. Transposon-mediated mutations of the 5'-UTR of the maize alcohol dehydrogenase *adh1* gene revealed the presence of both enhancer and inhibitor regions that act preferentially in pollen (Dawe et al., 1993). Transposon-mediated duplication of a 7-bp GGACTGA element within the *adh1* 5'-UTR gave rise to enhancement of *adh1* expression, which argues for a stimulatory role of this sequence on pollen gene expression. The GGACTGA element is also present in the 5'-UTR of four other *adh1* alleles, whereas a variant of the element (GGTCTGA) is located in the 5'-UTR of the pollen-expressed gene *bp19*. Although these studies have generated valuable data with regard to the role of the 5'-UTR of pollen transcripts in modulating gene expression in pollen, profound information about the presence, composition and mode of action of regulatory sequences is still scarce.

The aim of this thesis is to describe the role of the 5'-UTR of pollen-expressed genes in the regulation of pollen gene expression. Therefore, the research has been focused on two main objectives: a study of the functionality of pollen gene 5'-UTR sequences (**chapter 2** and **3**) and the identification of putative regulatory pollen gene 5'-UTR elements (**chapter 4** and **5**).

In order to obtain insight in the functionality of 5'-UTR sequences with regard to post-transcriptional regulation of gene expression in pollen, the research was initially focused on the pollen-expressed gene *ntp303* (**chapter 2** and **3**). In **chapter 2** is described to what extent the 5'- and 3'-UTR of *ntp303* contribute to direct pollen gene expression. Transient expression of several gene fusion constructs containing different combinations of promoter and UTR sequences linked to the open reading frame of a sensitive reporter gene was assayed in developing and germinating pollen. Furthermore, the importance of regulatory sequences in the *ntp303* 5'-UTR for modulation of pollen gene expression was established by means of 5'-UTR deletion analysis. **Chapter 3** reports the effect of the *ntp303* 5'-UTR on gene expression in stable tobacco transformants. Gene fusion constructs containing the *ntp303* promoter and different 5'-UTRs linked to the *luciferase* coding region were used to establish the activity of the *ntp303* 5'-UTR throughout pollen development and pollen tube growth. The sensitive *luciferase*$^{+}$ reporter gene enabled also to study to what extent the activity of the *ntp303* promoter and 5'-UTR was restricted to pollen. Furthermore, the stable transformants enabled to examine whether cellular or environmental conditions influenced the activity of the *ntp303* 5'-UTR.

In order to identify and characterize putative regulatory elements in the 5'-UTR of pollen-expressed genes, two different experimental approaches were followed (**chapter 4** and **5**). In **chapter 4** the isolation and characterization of a petunia homologue of *ntp303*, *php303* (*p303*) is described. Accumulation of *php303* mRNA and protein in developing and germinating petunia pollen was determined and compared with the expression kinetics of *ntp303* in tobacco. The activity of the *php303* 5'-UTR was assayed in petunia pollen tubes using the transient expression approach. To find conserved sequence regions, the 5'-UTR of *php303* was aligned with that of *ntp303*. Furthermore, gene datasets were searched for *php303* and *ntp303* homologous 5'-UTR sequences. Development of computational methods that identify short conserved sequences in large heterologous sequence datasets has enabled the identification of putative regulatory elements in co-regulated genes (Van Helden et al., 1998, 2000; Jacobs-Anderson and Parker, 2000). Since the existence of conserved 5'-UTR sequences over evolutionary distances may indicate an involvement of these sequences in the

regulation of pollen gene expression, a systematic statistical analysis has been performed in order to identify elements that are preserved in the 5'-UTR of pollen-expressed genes (**chapter 5**).

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Chapter 2

The 5'-untranslated region of the *ntp303* gene strongly enhances translation during pollen tube growth, but not during pollen maturation

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ABSTRACT

Transcripts of the *ntp303* gene accumulate abundantly throughout pollen development, whereas the protein only accumulates to detectable levels after pollen germination. In an attempt to explain the divergence in the accumulation profiles of the mRNA and the protein, we investigated the role of the untranslated regions (UTRs) in enhancing *ntp303* translation during the transition from developing to germinating pollen. *Luciferase* reporter gene fusion constructs containing the *ntp303* 5'-UTR gave rise to luciferase activity that was up to 60-fold higher during pollen tube growth than that of constructs containing different 5'-UTRs. No apparent differences in the luciferase activity of these constructs were observed during pollen development. The *ntp303* 5'-UTR-mediated increase in luciferase activity was not significantly influenced by coding region or 3'-UTR sequences. Furthermore, enhanced luciferase activity directed by the *ntp303* 5'-UTR occurred predominantly at the post-transcriptional level. A series of 5'-UTR deletion constructs was created to identify putative regulatory sequences required for the high level of translation during pollen tube growth. Two predicted stem loop structures (H-I and H-II) caused a complete inhibition of the enhanced translation after their total or partial deletion. A $(GAA)_8$ repeat within the H-I stem loop structure was demonstrated to be important for the modulation of translation efficiency. The H-II stem loop structure was found to be essential for the determination of mRNA stability.

INTRODUCTION

In plants as well as in animals, gamete development involves defined transitions of cells from one physiological state to another by mitotic and meiotic divisions. These series of events require multiple changes in gene expression. Many stages of gamete development in plant and animal species proceed almost without transcriptional activity and depend mainly upon translation of pre-synthesized mRNAs. Thus, in these species, post-transcriptional control of gene expression is very important for gamete development. Examples include the

post-transcriptional control of genes expressed during spermatogenesis in *Drosophila melanogaster* (Kuhn et al., 1991; Schäfer et al., 1993) and mouse (*Mus musculus*; Nayernia et al., 1994; Schäfer et al., 1995) and during oocyte development in marine invertebrates (Swenson et al., 1987) and mammals (Stutz et al., 1998; Lasko, 1999).

An example of post-transcriptional regulation of gene expression during gamete development in plants is the development and germination of the male gametophyte (pollen; for review, see Mascarenhas, 1990, 1993; McCormick, 1991, 1993; Taylor and Hepler, 1997). Pollen grains consist of a small generative cell and a large vegetative cell that are formed from microspores by a mitotic division (for review, see Mascarenhas, 1989; Bedinger, 1992). During subsequent pollen development, a range of processes leads to progressive dehydration of the grain and its transition to dormancy (Lin and Dickinson, 1984; Van Aelst et al., 1993). This maturation of pollen is generally accompanied by a progressive storage of large quantities of rRNAs, tRNAs, mRNAs and ribosomes (for review, see Mascarenhas, 1990, 1993). As soon as the pollen grain lands on a compatible stigma, an extensive rehydration of the grain occurs, leading to a rapid reactivation of the translation machinery that uses the stored RNAs. Proteins that are synthesized from these stored products are required for the progamic phase, i.e. germination and subsequent growth of the pollen tube (Mascarenhas, 1990, 1993; Muschietti et al., 1994).

Despite the importance of translation of pre-synthesized mRNAs in the contribution of sexual reproduction, little attention has been paid to elucidate the mechanisms underlying post-transcriptional regulation of pollen gene expression (Op den Camp and Kuhlemeier, 1998; Ylstra and McCormick, 1999; Honys et al., 2000). Many mRNAs from different eukaryotic systems can be modulated in their translation efficiency by signals encoded in the 5'- or 3'-untranslated region (UTR; for review, see Gallie, 1993, 1996; Fütterer and Hohn, 1996; Pain, 1996; Danon, 1997; Day and Tuite, 1998; Bailey-Serres, 1999). In these cases, translation has often been found to be regulated at the level of translation initiation. This led to the hypothesis that the UTRs might play an important role in the efficient induction of translation during the transition of developing pollen to pollen in the progamic stage (e.g. Bate et al., 1996). If so, it may be assumed that specific sequences within the 5'- or 3'-UTR are prerequisite to direct the high level of translation during pollen tube growth.

To obtain more insight in the mechanism of post-transcriptional regulation of pollen gene expression during pollen tube growth, we focused on the pollen gene *ntp303*. Regulation of the synthesis of the NTP303 protein takes place at the post-transcriptional level (Čapková

et al., 1994; Šťorchová et al., 1994; Wittink et al., 2000). Transcripts of the *ntp303* gene first appear after pollen mitosis I and continue to accumulate during pollen maturation and early stages of pollen tube growth (Weterings et al., 1992). In contrast, the protein only starts to accumulate at the onset of pollen rehydration (Wittink et al., 2000).

In the present study, we investigated the contribution of the UTRs of the *ntp303* gene in directing pollen gene expression. Several gene fusion constructs containing different promoter and UTR combinations linked to the *luciferase*$^{+}$ open reading frame were introduced in developing and germinating pollen by particle bombardment and their transient expression was assayed. Furthermore, several *ntp303* 5'-UTR deletion constructs were generated and tested to identify putative cis-acting regulatory sequences.

RESULTS

UTR gene fusion constructs

Several UTR gene fusion constructs containing the *ntp303* promoter, the firefly *luciferase*$^{+}$ coding region (Promega, Madison, WI), and different combinations of 5'- and 3'-UTRs were built to investigate the ability of the UTRs of *ntp303* to modulate translation during pollen development and pollen tube growth (Fig. 1A). The names of the constructs refer to their 5'- and 3'-UTRs (5'-UTR/3'-UTR). The abbreviation "35S" or "*R*" that is given in uppercase letters before a construct name indicates that the construct contains the CaMV 35S instead of the *ntp303* promoter or the *Renilla reniformis luciferase* instead of the firefly *luciferase* coding region, respectively.

The different constructs were introduced into developing or mature pollen by particle bombardment. The level of expression was estimated after a period of 20 h in vitro development or germination by measurement of luciferase activity. To correct for differences in bombardment efficiencies, a second construct was co-bombarded. This construct contained the *ntp303* promoter, a control 5'-UTR (syn44 5'), the *luciferase* reporter gene from *R. reniformis* and the CaMV termination sequence (35S 3'; ^{R}syn44 5'/35S 3'). The luciferase activity value of the firefly *luciferase* construct was normalized to the value of the *R.*

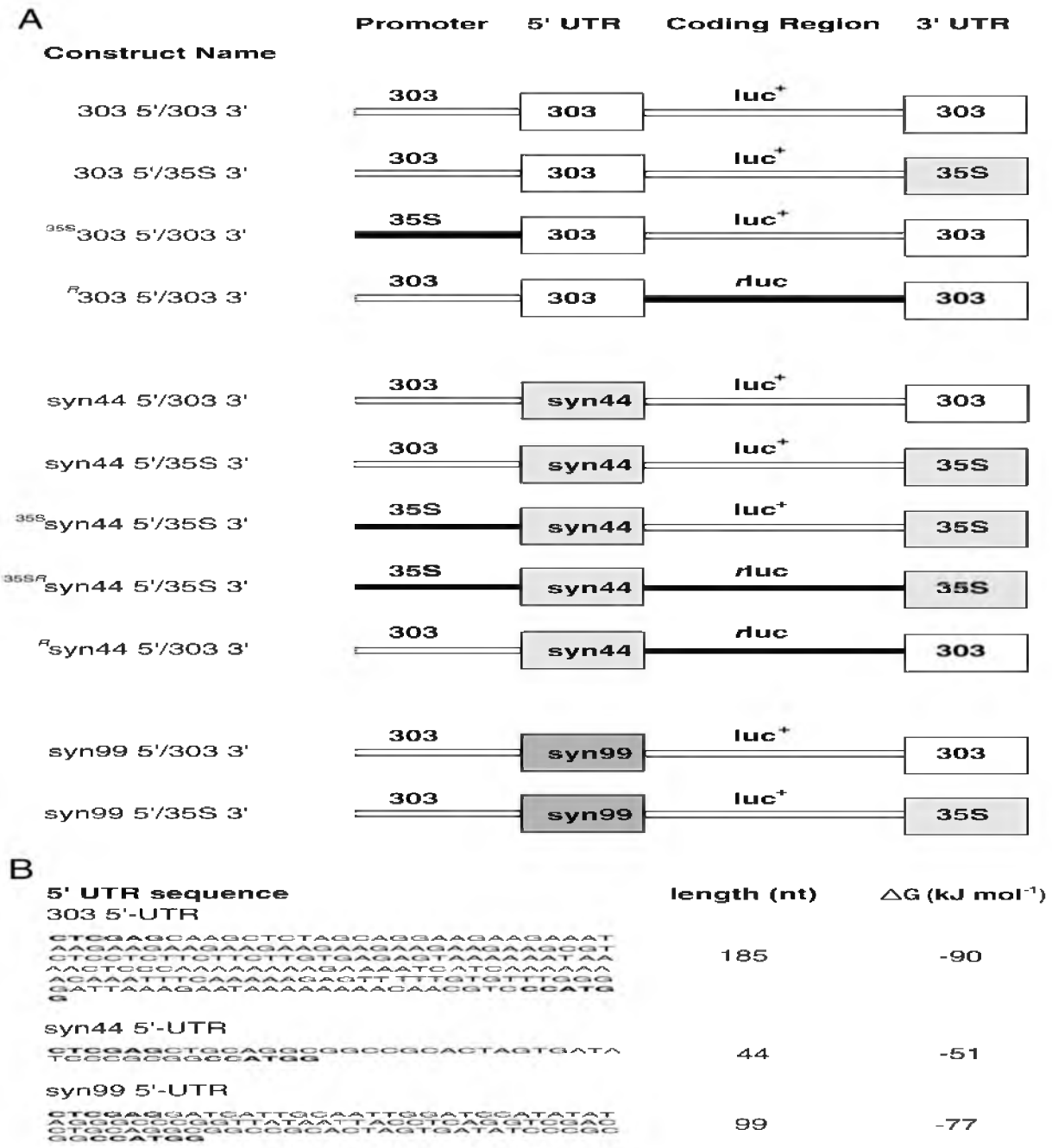

Figure 1. UTR gene fusion constructs used in the present study.

A, Schematic representation and names of the UTR gene fusion constructs. The constructs were driven by the *ntp303* (303) or the *cauliflower mosaic virus* (*CaMV*) *35S* (35S) promoter. The coding regions that were used in the constructs are firefly *luciferase* (luc^{+}) or *Renilla reniformis luciferase* (rluc). The left or right box in the construct represents the 5'- or 3'-UTR, respectively. UTR abbreviations: 303, *ntp303* 5'- or 3'-UTR; syn44, control 44 5'-UTR; syn99, control 99 5'-UTR; 35S, *CaMV 35S* 3'-UTR. The construct name refers to the 5'- and 3'-UTRs (5'-UTR/3'-UTR). "35S" or "*R*" in uppercase before a construct name means that the construct contains the *CaMV 35S* promoter or the *R. reniformis luciferase* coding region, respectively. B, Sequence, length, and the calculated stability of the 5'-UTRs used in the UTR gene fusion constructs.

reniformis luciferase construct, which gave rise to the relative luciferase activity. For each construct, at least six independent bombardments were performed.

The effect of *ntp303* UTRs on luciferase activity during pollen development and pollen tube growth was determined by comparing the translation level of constructs containing different combinations of *ntp303* and control UTRs. For replacement of the *ntp303* 3'- or 5'-UTR, we used the CaMV 35S termination sequence (35S 3') or two different control leader sequences designated as syn99 5' and syn44 5' (Fig. 1B). UTR gene fusion constructs containing the control 5'-UTRs have been demonstrated to be translated efficiently by Bate et al. (1996). The syn99 5'-UTR was used as a control UTR because it revealed a free energy value that was more or less comparable with that of the *ntp303* 5'-UTR (calculated energy values [ΔG] of –90 and –77 kJ mol^{-1}, respectively; Fig. 1B). Lowering the potential energy (i.e. a more negative value of ΔG) of secondary structures within a 5'-UTR has a negative effect on translation (Kozak, 1989; Gallie et al., 2000). The syn44 5'-UTR was used as a positive control because its secondary structure has a relative high potential energy (ΔG of –51 kJ mol^{-1}), and therefore a positive effect on translation compared with the *ntp303* and syn99 5'-UTRs. The difference in translation efficiency of both control UTRs becomes clear in Figure 2, A and B. During pollen development and pollen tube growth, the construct containing the syn44 5'-UTR gave rise to an approximately 10-fold higher luciferase activity level compared with the syn99 5'-UTR-containing construct.

The 5'-UTR, but not the 3'-UTR, of *ntp303* enhances translation specifically during pollen tube growth

The effect of *ntp303* UTRs on luciferase activity during pollen development and pollen tube growth was investigated by comparing the expression level of a construct containing the *ntp303* UTRs (303 5'/303 3') with that of constructs containing control UTRs (the syn99 5' or syn44 5'-UTR and the 35S 3'-UTR). In developing pollen incubated for 20 h after bombardment, the *ntp303* UTRs construct gave rise to a luciferase activity level that was approximately 4-fold higher than that of syn99 5'/35S 3' and slightly lower than that of syn44 5'/35S 3' (Fig. 2A). After 20 h of pollen tube growth, the luciferase activity level of 303 5'/303 3' was approximately 60- and 6-fold higher than that of syn99 5'/35S 3' and syn44 5'/35S 3', respectively (Fig. 2B). The differences in the luciferase activity level could already be observed in pollen tubes 5 h after bombardment (data not shown). The luciferase activity

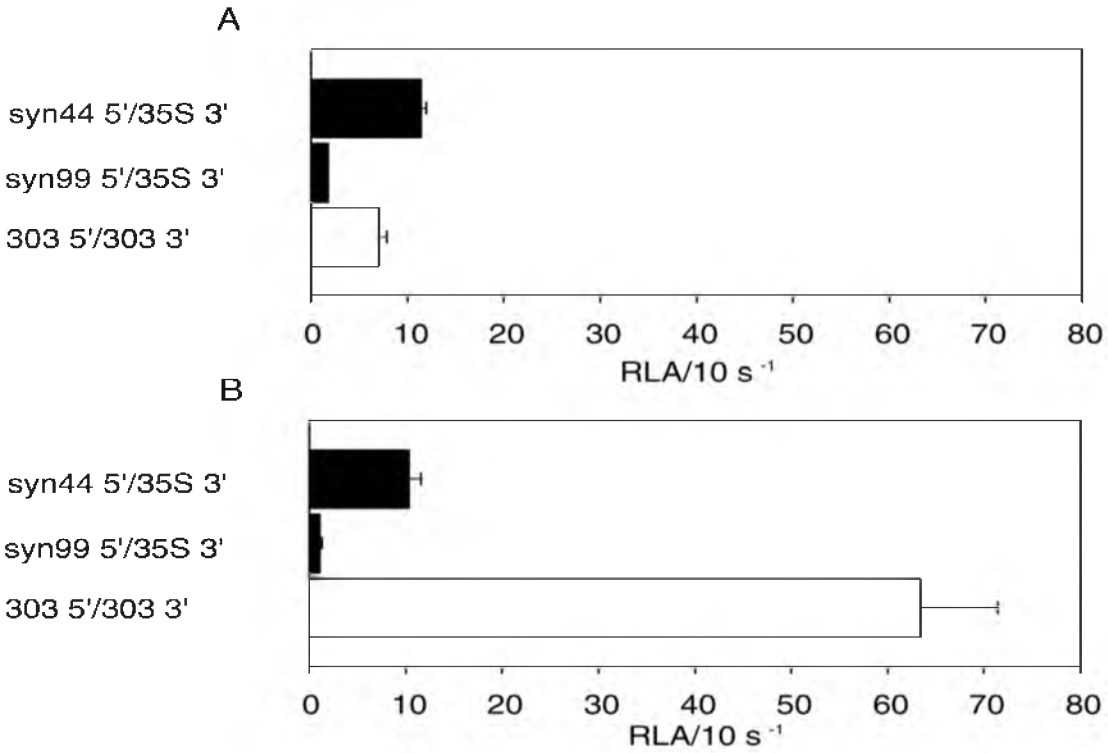

Figure 2. Luciferase activity of gene fusion constructs in developing pollen (A) and growing pollen tubes (B) containing control (black) or *ntp303* (white) UTRs.

RLA/10 s^{-1} means the relative luciferase activity (luminescence) per 10-s measuring time after normalization with the luciferase activity of the reference construct ^{R}syn44 5'/35S 3'. Results are given as means ± SE ($n \geq 6$). For details, see "Results" and "Materials and Methods".

levels of the constructs containing the control UTRs were not significantly different during pollen development or pollen tube growth (Fig. 2, A and B). This clearly illustrates that expression mediated by these control UTRs is independent of the developmental phase in which they were tested.

To examine whether the 5'-UTR or the 3'-UTR of the *ntp303* mRNA determines the level of expression during pollen development and pollen tube growth, luciferase activity of gene fusion constructs containing the *ntp303* 5'- and 35S 3'-UTRs or the syn44 5'- and *ntp303* 3'-UTRs was compared with that of syn44 5'/35S 3'. No significant differences in the luciferase activity level of the *ntp303* UTR and control UTRs-containing constructs were observed during pollen development (Fig. 3A). During pollen tube growth, the *ntp303* 5'-UTR increased the activity of luciferase to a level that was almost 8-fold higher than that of the control 5'-UTR construct (Fig. 3B). This enhancement effect was absent in the *ntp303* 3'-UTR construct.

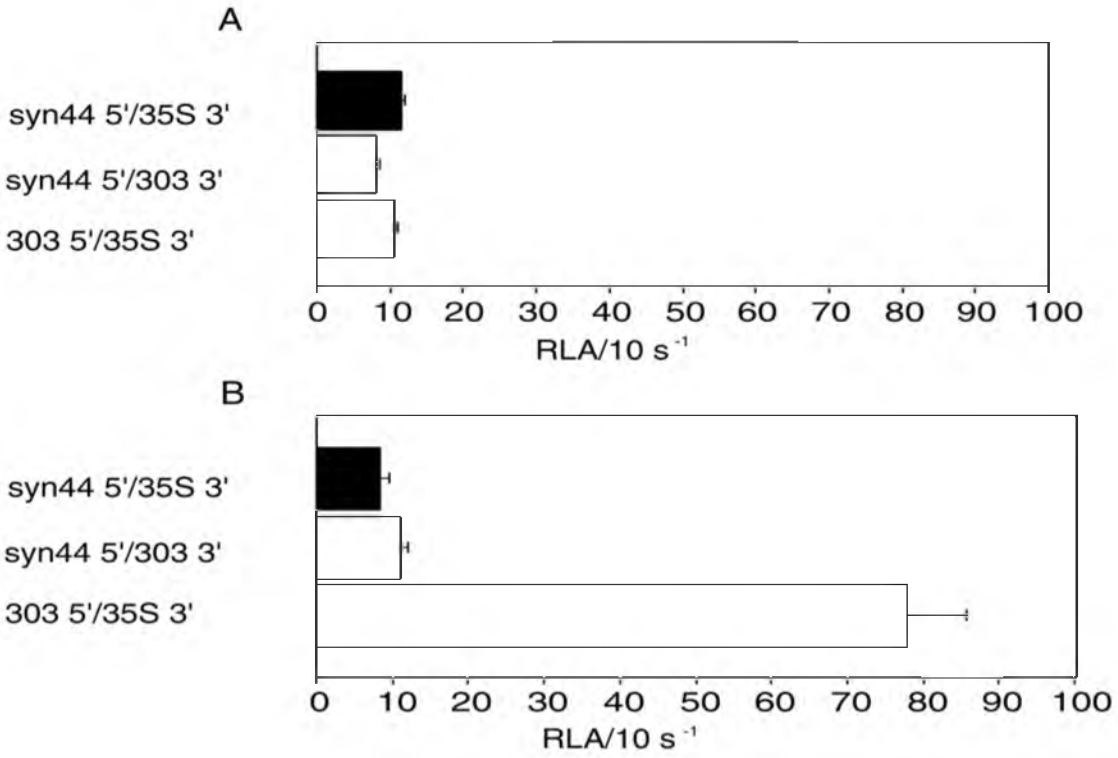

Figure 3. Luciferase activity of gene fusion constructs in developing pollen (A) and growing pollen tubes (B) containing control (black) or combinations of control and *ntp303* (white) UTRs.

Activity of the firefly luciferase determined for the test constructs was normalized with the *R. reniformis* luciferase activity of the reference construct ^{R}syn44 5'/35S 3' (RLA/10 s^{-1}). Results are given as means ± SE ($n \geq 6$).

To exclude the possibility that the enhancement of luciferase activity mediated by the *ntp303* 5'-UTR in growing pollen tubes was the result of a specific interaction between the 5'-UTR and the firefly *luciferase* coding region, this coding region was replaced by the *R. reniformis luciferase* coding region in the constructs syn44 5'/35S 3' and 303 5'/303 3'. The firefly and the *R. reniformis luciferase* mRNAs exhibit no significant sequence identity with each other. Normalization of the luciferase activity of these constructs was established by co-bombardment with a construct containing the syn44 5'-UTR, the firefly *luciferase* coding region, and the 35S termination sequence. As shown in Figure 4, the *ntp303* UTRs gave rise to a luciferase activity level that was approximately 7-fold higher than that of the control UTRs. Because this enhancement effect of the *ntp303* 5'-UTR was also found for firefly *luciferase* mRNAs, this suggests that there is no specific interaction between the *ntp303* 5' UTR and the coding region.

We conclude that the 5'-UTR of the *ntp303* gene is an important determinant in the high level of expression during pollen tube growth.

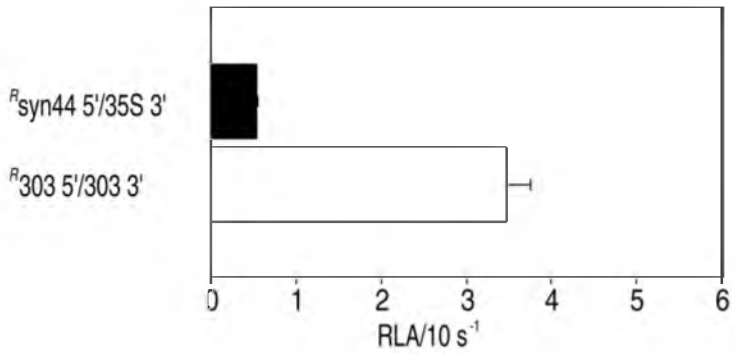

Figure 4. Transient expression of gene fusion constructs containing the *R. reniformis luciferase* coding region and control (black) or *ntp303* (white) UTRs in growing pollen tubes.

Activity of the *R. reniformis* luciferase determined for the test constructs was normalized with the firefly luciferase activity of the reference construct syn44 5'/35S 3' (RLA/10 s^{-1}). Results are given as means ± SE ($n \geq 6$).

The 5'-UTR-specific enhancement during pollen tube growth is post-transcriptional

To investigate whether the enhancement mediated by the *ntp303* 5'-UTR during pollen tube growth was the result of post-transcriptional regulation or an enhanced transcript level, we determined the relative transcript and luciferase activity levels of pollen that were bombarded with the syn99 5'/35S 3', 303 5'/35S 3' or syn99 5'/303 3' construct (Table I). Each pollen batch bombarded was separated in two fractions. One fraction was used for total RNA isolation and the other was used for the luciferase assay. Ten micrograms of total RNA was hybridized with a ^{32}P-labeled *luciferase* probe. The relative transcript level was determined by calculation of the ratio of the hybridization signal of firefly *luciferase* mRNA to that of *R. reniformis luciferase* mRNA of the co-bombarded ^{R}syn44 5'/35S 3' construct. The *ntp303* 5'-UTR construct showed a relative transcript level that was approximately 2- to 3-fold higher after 20 h of pollen tube growth than that of the syn99 5'-UTR construct. The construct containing the *ntp303* 5'-UTR exhibited a 50-fold increase in luciferase activity as compared to syn99 5'/35S 3' (Table I). These data indicate that during pollen tube growth, chimeric *luciferase* transcripts containing the *ntp303* 5'-UTR are translated more efficiently than *luciferase* mRNAs containing the control 5'-UTR. Although the high luciferase activity

levels are primary due to an enhanced translation efficiency, the *ntp303* 5'-UTR exhibits a stimulatory influence on the transcript level.

Construct	Relative *luc* mRNA abundance (counts)	Relative LUC activity
syn99 5'/35S 3'	1.36 ± 0.17 (1.00)	1.02 ± 0.21 (1.00)
303 5'/35S 3'	3.70 ± 0.22 (2.72)	50.60 ± 0.22 (49.61)
syn99 5'/303 3'	1.81 ± 0.22 (1.33)	2.50 ± 0.42 (2.45)

Table I. Analysis of the relative transcript (relative *luciferase* mRNA abundance) and luciferase activity (relative luciferase activity/10 s^{-1}) levels of UTR gene fusion constructs during pollen tube growth.

The values in parentheses represent the fold increase of the transcript and luciferase activity levels compared to the levels of the syn99 5'/35 3' construct. Results are given as means ± SE ($n \geq 6$). See "Results" for a description of the followed methodology. RLA, relative luciferase activity.

The 5'-UTR-mediated enhancement of translation also occurs in sporophytic tissue, but is highest in pollen tubes

To test whether the *ntp303* 5'-UTR-mediated enhancement of translation in growing pollen tubes was restricted to a pollen-specific environment, the constructs syn44 5'/35S 3' and 303 5'/303 3' were reconstructed by replacing the *ntp303* promoter with the *CaMV 35S* promoter. The *CaMV 35S* promoter is somewhat active in pollen, and highly active in sporophytic tissues (Twell et al., 1989). After bombardment of these constructs into mature pollen and young leaves followed by 20 h of in vitro incubation, luciferase activity was assayed. Normalization of the luciferase activity level of these constructs was established by co-bombardment with a construct containing the *CaMV 35S* promoter, the syn44 5'-UTR, the *R. reniformis luciferase* reporter gene, and the *35S* 3'-UTR. In growing pollen tubes, the *ntp303* 5'-UTR increased the luciferase activity approximately 5-fold (Fig. 5A). The difference in the luciferase activity level approached that of the constructs containing the same UTR combinations but linked to the *ntp303* promoter (compare Fig. 5A with Fig. 2B). In young leaves, the *ntp303* 5'-UTR construct led to a luciferase activity level that was approximately 2-fold higher than the control UTR's construct (Fig. 5B). These data demonstrate that the *ntp303* 5'-UTR-mediated enhancement of translation may also occur in sporophytic cells.

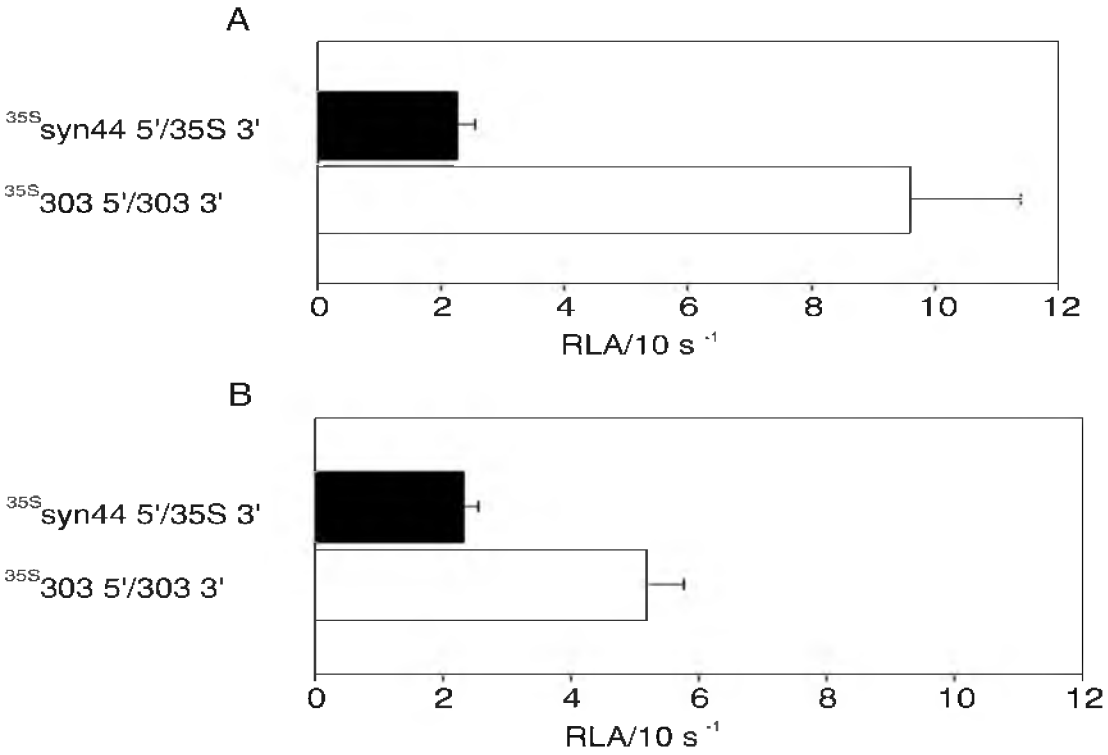

Figure 5. Luciferase activity of gene fusion constructs containing the *CaMV 35S* promoter in growing pollen tubes (A) and young leaves (B).

Key to bars: black is the expression of ^{35S}syn44 5'/35S 3', and white is the expression of ^{35S}303 5'/303 3'. RLA/10 s^{-1} indicates the relative luciferase activity per 10-s measuring time after normalization with the luciferase activity of the reference construct ^{35SR}syn44 5'/35S 3'. Results are given as means ± SE ($n \geq 6$).

Enhancement of translation during pollen tube growth can be attributed to specific regions within the *ntp303* 5'-UTR

Figure 6 illustrates the predicted secondary structure of the *ntp303* 5'-UTR as analyzed with the RNAdraw software package (Matzura and Wennborg, 1996). There are two predicted stem loop structures designated H-I and H-II. The H-I stem loop structure is located at the 5'-terminus and has a ΔG value of -64 kJ mol^{-1}. This structure contains eight repeats of a GAA triplet in the external loop. The H-II structure is located 22 nucleotides upstream from the translation initiation site and has a ΔG of -26 kJ mol^{-1}. The effect of sequences within the H-I and H-II structures on enhancement of translation during pollen tube growth was investigated by a series of *ntp303* 5'-UTR deletion constructs (Figs. 7A and 8A). These constructs were bombarded into mature pollen and the luciferase activity was assayed after 20 h of pollen tube growth (Figs. 7B and 8B). Figure 7B shows the luciferase activity of UTR gene fusion constructs with deletions within the H-I stem loop structure. The lowest

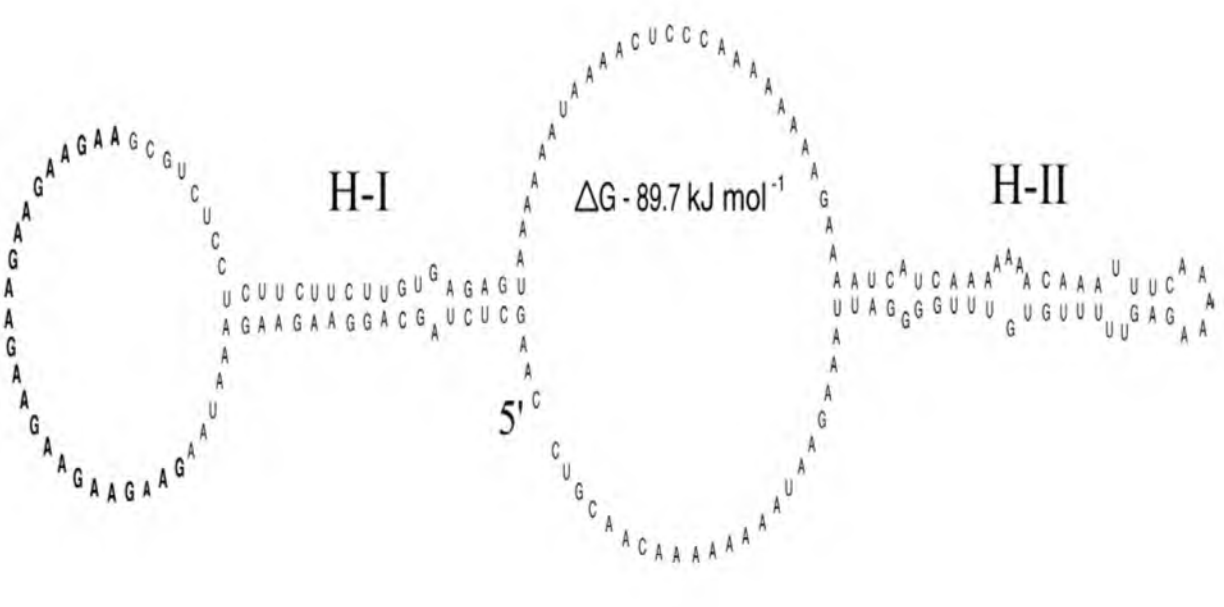

Figure 6. Predicted secondary structure of the *ntp303* 5'-UTR.

Structure prediction was performed using the RNAdraw software package (Matzura and Wennborg, 1996). H-I and H-II represents two predicted stem loop structures. The H-I structure contains eight repeats of a GAA triplet in the external loop (bold). The start of the transcription initiation site is indicated by 5'.

level of luciferase activity was found after internal deletion of the $(GAA)_8$ repeat (ΔGAA 303 5'/35S 3'). This luciferase activity level was comparable with the level of the control construct containing the syn99 5'-UTR (data not shown). A decrease in luciferase activity of approximately 94 % occurred after deletion of the first 55 nucleotides (Δ55 303 5'/35S 3') at the 5'-terminus including the $(GAA)_8$ repeat. Deletion of the first 29 nucleotides at the 5'-terminus of the *ntp303* 5'-UTR (Δ29 303 5'/35S 3') caused only a slight decrease in luciferase activity compared with that of the unmodified *ntp303* 5'-UTR. An almost complete inactivation of reporter gene activity was achieved after deletion of the last 70 nucleotides at the 3'-terminus of the *ntp303* 5'-UTR, which included the complete H-II structure (Δ70 303 5'/35S 3'; Fig. 8B). The same was true after internal deletion of only the H-II structure (ΔH-II 303 5'/35S 3'). In both cases, the luciferase activity values were in the same range as the background values (i.e. the measured autoluminescence of the luciferin substrate). Although the predicted stem loop structures have not been confirmed by nuclease-sensitive site mapping, these results clearly indicate that specific sequence regions within these putative structures are essential for enhancement of translation during pollen tube growth.

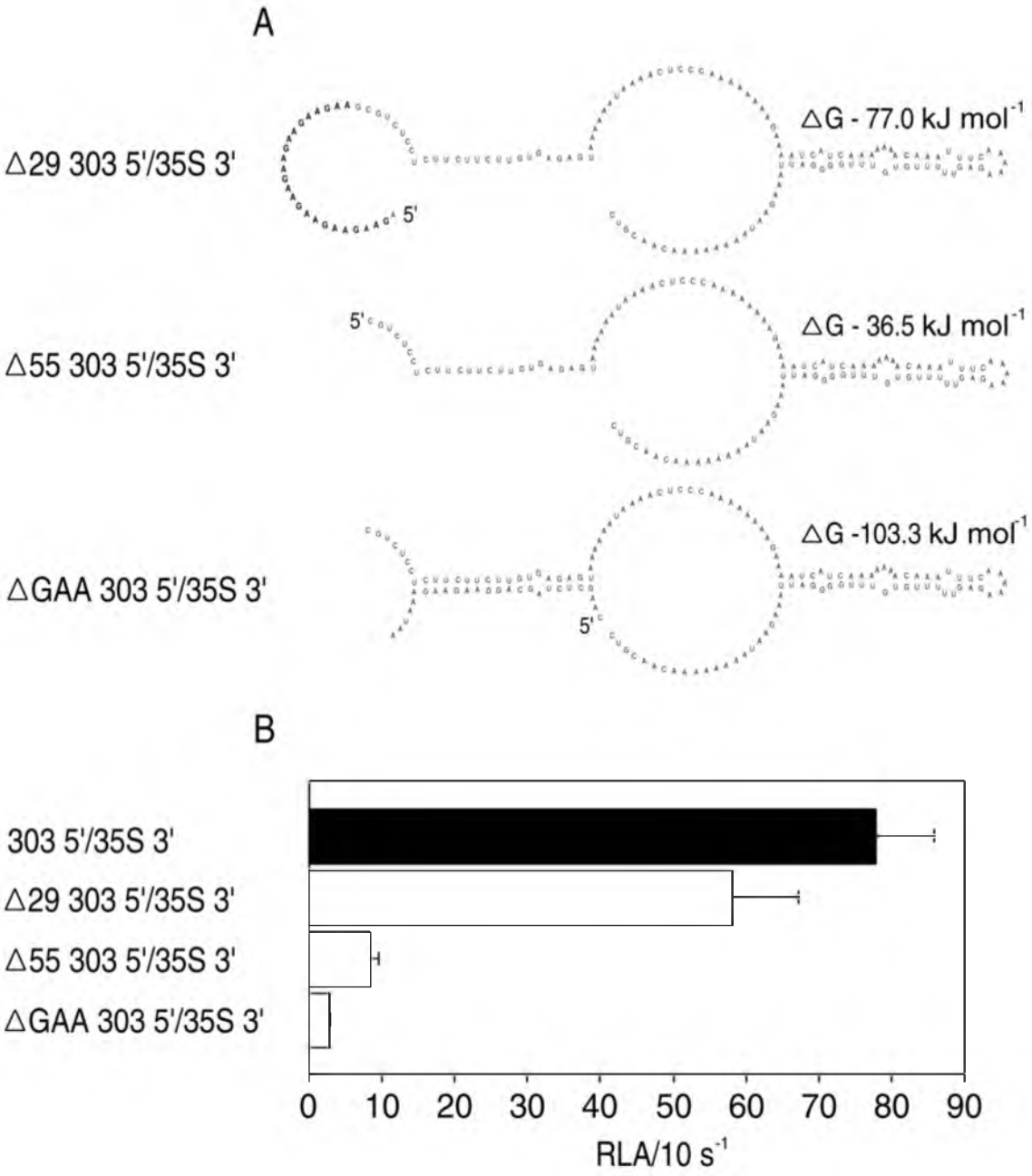

Figure 7. The effect of deletions in the H-I stem loop structure of the *ntp303* 5'-UTR on luciferase activity.

A, Graphic representation of the *ntp303* 5'-UTR with different H-I deletions. The secondary structures were predicted and the enthalpy calculated using the RNAdraw software package (Matzura and Wennborg, 1996). Internal deletion of 25 nucleotides including a $(GAA)_8$ repeat gave rise to the ΔGAA 303 5'/35S 3' construct. Deletion of the first 29 or 55 nucleotides at the proximal end of the *ntp303* 5'-UTR gave rise to the Δ29 303 5'/35S 3' or Δ55 303 5'/35S 3' construct, respectively. The start of the 5'-UTR is indicated by 5'. B, Luciferase activity of the construct containing the *ntp303* 5'- and *CaMV 35S* 3'-UTRs (black) and the H-I deletion constructs (white) in pollen tubes. Results are given as means ± SE ($n \geq 6$). Details about the experimental procedure are given in "Results" and "Material and Methods".

The predicted H-I and H-II structures in the *ntp303* 5'-UTR influence transcript accumulation and translation efficiency

Whether the decrease in translation of the 5'-UTR deletion constructs was the result of a change in the transcript level or translation efficiency was investigated by measuring the relative transcript and luciferase activity levels of some of the *ntp303* 5'-UTR deletion constructs (Table II). The relative transcript values of the constructs containing deletions of the complete H-II structure (Δ70 303 5'/35S 3' and ΔH-II 303 5'/35S 3') dropped to a level that was more than 2.5-fold lower than that of the construct containing the unmodified *ntp303* 5'-UTR. Internal deletion of the $(GAA)_8$ repeat (ΔGAA 303 5'/35S 3') resulted in a relative transcript level that was somewhat lower than the transcript level of 303 5'/303 3'. In contrast to the effects of either the deletion of the $(GAA)_8$ repeat or the H-II structure on the relative transcript level, a more drastic effect was observed for the luciferase activity levels. A drastic decrease in luciferase activity was observed after deletion of the H-II structure, and the measured values were in the range of the luciferin autoluminescence background. Deletion of the $(GAA)_8$ repeat revealed an almost 2-fold lower luciferase activity value compared with 303 5'/303 3'. From these data, we conclude that the drop in translation observed after deletion of either the H-I or H-II structures is the result of a decrease in the transcript level, but most drastically in the translation efficiency.

DISCUSSION

In the present study, we investigated the mechanism underlying 5'-UTR-mediated enhancement of translation in pollen. Although the transient expression results have to be confirmed by stable transformants, we conclude that the 5'-UTR of the *ntp303* gene exhibits the capacity to enhance translation (measured as luciferase activity) in pollen tubes, where it acts as an autonomous enhancer element independent of linked promoter, coding region, or 3'-UTR sequences. Specific sequences within two predicted stem loop structures of the 5'-UTR are essential for enhanced translation. Enhancer activity mediated by the 5'-UTR is absent during pollen development and appears specifically during pollen tube growth.

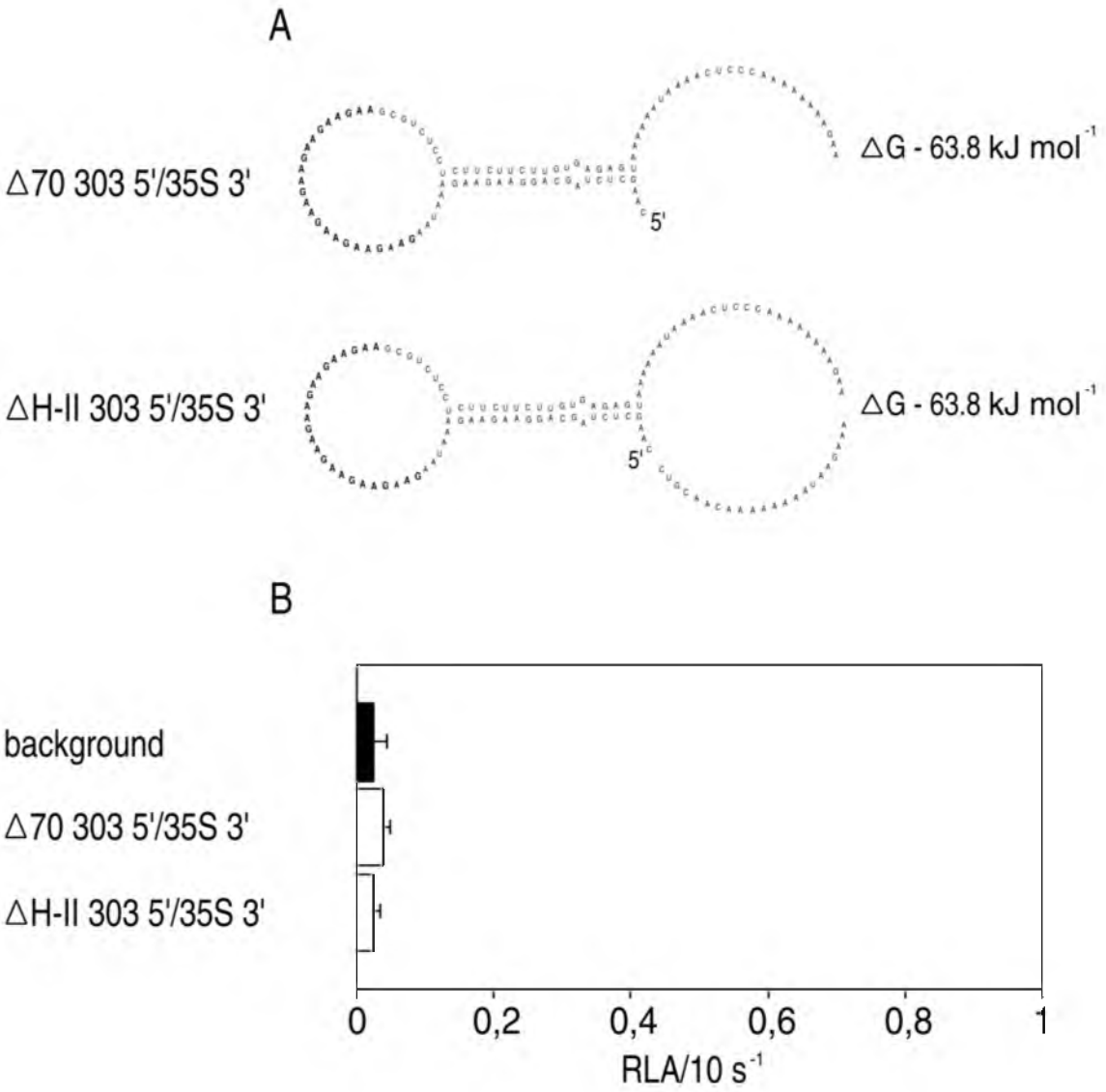

Figure 8. Effect of H-II stem loop structure deletions within the *ntp303* 5'-UTR on luciferase activity.

A, Graphic representation of the *ntp303* 5'-UTR with different H-II deletions. Secondary structure prediction and enthalpy calculation was performed with the RNAdraw software package (Matzura and Wennborg, 1996). Deletion of 70 nucleotides at the 3'-terminus of the *ntp303* 5'-UTR gave rise to the Δ70 303 5'/35S 3' construct. Internal deletion of the H-II structure gave rise to the ΔH-II 303 5'/35S 3' construct. The start of the 5'-UTR is indicated by 5'. B, Autoluminescence of the luciferin substrate (black) and luciferase activity of the H-II deletion constructs (white) in pollen tubes. Results are given as means ± SE ($n \geq 6$). See "Results" and "Material and Methods" for details about the experimental procedure.

The enhancement effect of the *ntp303* 5'-UTR is not limited to pollen tubes but also can be found in sporophytic tissues.

In growing pollen tubes, the high level of translation of *ntp303* transcripts is fully dependent on the 5'-UTR. The presence of the element led to activity values of two reporter

genes to a level up to 60-fold higher than that of constructs containing control 5'-UTRs (syn44 5' and syn99 5'; Fig. 2B). The enhancement effect is not the result of a structurally inefficient translation of the control constructs because in this case the *ntp303* 5'-UTR would lead to a luciferase activity level that is similar in developing pollen, growing pollen tubes, and leaves. The similar ratios of luciferase activity levels for the control constructs during pollen development and pollen tube growth (Fig. 2, A and 2B) argue also against their inefficient translation.

Comparison of the relative transcript and luciferase activity values mediated by the *ntp303* 5'-UTR or the control UTRs revealed that the enhancement is mainly the result of an increase in the translation efficiency (Table I). In line with this, the level of enhancement is only slightly influenced by the strength of the promoter, which is clear from the activities of the constructs containing the *CaMV 35S* promoter (Fig. 5A). That the 5'-UTR may be a regulatory site in the modulation of translation fits the scanning model of protein synthesis in which the pre-initiation complex scans the 5'-UTR in search of the first translation initiation codon (Kozak, 1999). Although the *ntp303* 5'-UTR acts mainly at the translational level, an increase in the relative transcript level was also observed. Whether the increase of the relative transcript level was the result of an increase in the transcription rate or transcript stability remains to be investigated. We assume that the 5'-UTR affects the transcript stability because the enhancement of translation is rather independent of the transcriptional activity of the linked promoter.

The regulatory effect of the *ntp303* 5'-UTR is also independent of the linked coding region and 3'-UTR sequences (Figs. 3B and 4). Therefore, the *ntp303* 5'-UTR acts as an autonomous element in the modulation of translation. Furthermore, this autonomy is also apparent in sporophytic tissue, although the difference is larger in growing pollen tubes, which argues for the involvement of pollen tube-preferential factors.

Because the action of the *ntp303* 5'-UTR is independent of other gene constituents, we hypothesize that the *ntp303* 5'-UTR contains sequence elements or secondary structures that are essential for the regulatory effect. The presence, position, and architecture of secondary structures and the nature of primary sequences have been demonstrated to modulate the level of translation efficiency of mRNAs in higher eukaryotes (Kozak, 1989; Bailey-Serres and Dawe, 1996; Klaff et al., 1996; Curie and McCormick, 1997; Gallie et al., 2000). We created several deletions within the *ntp303* 5'-UTR to identify putative regulatory elements in the *ntp303* 5'-UTR (Figs. 7A and 8A). Deletions of the predicted H-II stem loop structure in the 5'-UTR resulted in a strong decrease in luciferase activity during pollen tube growth (Fig.

8B). Determination of the relative transcript levels also revealed a strong decrease in transcript accumulation (Table II). We assume that deletion of the H-II stem loop structure

Construct	Relative *luc* mRNA abundance	Relative LUC activity
	counts	RLA/10 s^{-1}
303 5'/303 3'	2.79 ± 0.09 (1.00)	31.08 ± 5.61 (1.00)
ΔGAA 303 5'/35S 3'	2.30 ± 0.51 (0.77)	17.49 ± 4.76 (0.56)
Δ70 303 5'/35S 3'	1.13 ± 0.06 (0.38)	0.01 ± 0.00 (0.00)
ΔH-II 303 5'/35S 3'	0.82 ± 0.09 (0.28)	0.01 ± 0.00 (0.00)

Table II. Effect of different *ntp303* 5'-UTR deletions on the relative transcript (relative *luciferase* mRNA abundance) and luciferase activity (relative luciferase activity/10 s^{-1}) levels of different constructs during pollen tube growth.

The values in parentheses represent the fold increase of the transcript and luciferase activity levels compared with the levels of the 303 5'/303 3' construct. Results are given as means ± SE ($n \geq 6$). For details, see "Results". RLA, relative luciferase activity.

predominantly affects mRNA stability because the *ntp303* 5'-UTR-mediated translation enhancement is mainly independent of the linked *ntp303* promoter. Although the decrease in the transcript level could not completely account for the drop of translation of the H-II deletion constructs, we conclude that the H-II stem loop structure contains sequence elements that are important for the determination of *ntp303* mRNA stability. It is known that 5'-UTRs can modulate mRNA stability in plants (Dickey et al., 1998; Anderson et al., 1999; Nickelsen et al., 1999; Hua et al., 2001). Like the H-II deletions, removal of the predicted H-I stem loop structure also caused a strong decrease in the luciferase activity level during pollen tube growth (Fig. 7B). The strongest effect on the luciferase activity level was established after internal deletion of the $(GAA)_8$ repeat. Unlike the H-II deletions, deletion of the $(GAA)_8$ repeat caused only a slight decrease in the relative transcript level (Table II). From this, we conclude that the H-I stem loop structure contains sequence elements that are important for the modulation of translation efficiency. The decrease in the luciferase activity level after deletion of (parts of) the H-I stem loop structure are in contradiction to the generally accepted view that removal of secondary structures within the 5'-UTR often results in higher translation by facilitating scanning (Kozak 1989; Gallie et al., 2000). This indicates that primary sequences within the H-I stem loop, rather than structural characteristics of the

ntp303 5'-UTR, determine the regulatory effect. This conclusion is strengthened by the observation that internal deletion of the $(GAA)_8$ repeat in the H-I structure, which causes a minor alteration in the overall ΔG value of the *ntp303* 5'-UTR, led to complete inhibition of the enhanced translation. The $(GAA)_8$ repeat clearly represents a primary sequence element within the *ntp303* 5'-UTR that is necessary for enhancement of translation during pollen tube growth.

In contrast to the effect of the *ntp303* 5'-UTR in pollen tubes and sporophytic cells, no enhancement of luciferase activity was observed in developing pollen (Fig. 2A). This implies that, although *ntp303* transcripts accumulate in developing pollen, the presence of the 5'-UTR is not sufficient to induce a high level of translation of these transcripts. Because the enhancement effect was already observed during early phases of pollen tube growth, it seems obvious that conditions at the start of pollen tube growth define the onset of the activity of the *ntp303* 5'-UTR. The temporal activity of the *ntp303* 5'-UTR differs from that of another pollen-expressed gene, *lat52*. Here, the 5'-UTR increased luciferase activity already during pollen development (Bate et al., 1996).

With regard to mechanisms that account for the role of the *ntp303* 5'-UTR in regulation of translation of *ntp303* transcripts during the transition of developing to germinating pollen, we propose the following model. Pollen development and pollen tube growth are two physiologically distinct phases in the life span of the pollen grain. In the final phase of development, progressive dehydration transforms the developing pollen grain into a dormant structure that contains a large stock of pre-synthesized rRNAs, tRNAs, ribosomes, and mRNAs (for review, see Mascarenhas, 1990, 1993). To avoid premature degradation, it is obvious that these stored transcripts exhibit a high degree of stability. *Ntp303* transcripts have been shown to be highly stable during pollen development (Ylstra and McCormick, 1999). The drastic decrease in the transcript level after deletion of the H-II stem loop structure strongly suggests that parts of the *ntp303* 5'-UTR are involved in stabilization of *ntp303* transcripts that are utilized during subsequent pollen tube growth. Delayed translation of stored *ntp303* transcripts has been experimentally demonstrated (Čapková et al., 1994; Štorchová et al., 1994; Wittink et al., 2000). Rehydration of the mature pollen grain leads to (re-)initiation of translation. In such a case, the translation machinery must select transcripts that code for products needed for pollen germination and tube growth from the total mRNA population. It may be assumed that such a preferential translation of transcripts occurs through interaction of specific factors with selected 5'-UTRs. Such a mechanism would explain the enhancement of translation mediated by the *ntp303* 5'-UTR during pollen tube

growth. The decrease in translation after deletion of (parts of) the H-I stem loop structure within the 5'-UTR might be due to removal of sequences that are crucial for the interaction with these "enhancer" factors. It is plausible that other pollen-expressed genes that code for products that are needed for pollen germination or tube growth are regulated by a similar mechanism. Examination of the architecture of 5'-UTRs of other pollen genes by computer analysis is suitable to investigate this possibility. A first attempt has been made to address this question by means of computational pattern discovery (chapter 5, this thesis).

The key function of the 5'-UTR of *ntp303* transcripts to direct phase-dependent protein synthesis exhibits parallels with translation regulation mechanisms in reproduction processes in animals and other plant systems. Examples are enhanced translation of transcripts during spermatogenesis (Schäfer et al., 1993; Nayernia et al., 1994; Gu and Hecht, 1996), oocyte development (Lasko, 1999), and gametophyte development (Bate et al., 1996). As in pollen, changes in the activity of 5'-UTRs of genes that are under post-transcriptional control in these systems often occur during transition of tissues or cells to another physiological state. In this respect, it would be intriguing to examine whether components of the regulatory mechanisms of translation of stored pollen transcripts are conserved in other reproduction systems.

MATERIALS AND METHODS

Plant material

Greenhouse-grown plants of tobacco (*Nicotiana tabacum* L. cv Petit Havana SR1) were used as the source of pollen and leaf tissue for microprojectile bombardment. To assess the translation of the different UTR gene fusion constructs during pollen development, immature pollen of the late-bicellular stage were aseptically isolated from flower buds of 35 mm length in M1 medium as previously described (Tupý et al., 1991). Translation of the UTR gene fusion constructs during pollen tube growth was measured using mature pollen that was isolated from dehiscent tobacco flowers (Van Herpen et al., 1992). After isolation, the pollen pellet was suspended in 100 µL M1 medium at a density of 10^8 cells mL^{-1}. To fixate the pollen for particle bombardment, the pollen suspension was pipetted onto the surface of a

sterile Hybond-N$^+$ membrane (Amersham, Buckinghamshire, UK) that was placed on 1% (w/v) agar-solidified M1 medium. Following bombardment, the membrane containing late-bicellular or mature pollen was soaked in 10 mL of M1 medium or Read medium (Read et al., 1993a, 1993b), respectively. The late-bicellular pollen was incubated at 25°C in the dark at vigorous shaking. After centrifugation, the mature pollen was suspended in a 10-mL tube containing 0.5 mL Read medium followed by a 20-h incubation in the dark at 25°C. Treatment of leaf tissue before and after bombardment was performed as described by Hamilton et al. (1992). In all cases, bombardments were performed within 60 min of placing the plant material onto the solidified medium.

Preparation of gene fusion constructs containing different UTRs

In all constructs, either a modified version of the firefly *luciferase* coding region (luc$^+$), or a *luciferase* coding region from *Renilla reniformis* (rluc) was used as the reporter gene (Fig. 1A). The *luciferase*$^+$ coding region was amplified by PCR on the pGL3 vector (Promega) using a forward sequence-specific primer which introduced a *Nco*I site at the 5'-end (5'-ATATCCATGGAAGACGCC; *Nco*I site underlined) and a reverse sequence specific primer which introduced a *Bam*HI site at the 3'-end (5'-ATATGGATCCTTACACGGCGATC; *Bam*HI site underlined). The *R. reniformis luciferase* coding region was amplified by PCR on the pRL-SV40 vector (Promega) using the following sequence-specific primers: 5'-GTGTCCATGGATGACTTCGAAAG (*Nco*I site underlined) and 5'-GTGTGGATCCTTATTGTTCATTTTTGAG (*Bam*HI site underlined). For construction of ^{35S}syn44 5'/35S 3', the PCR product of *luciferase*$^+$ was digested with *Nco*I and *Bam*HI and, after removal of the *luciferase* coding region, ligated into the *Nco*I and *Bam*HI sites of pRTS2LUC (Bate et al., 1996). pRTS2LUC contains the *CaMV 35S* promoter (Wilkinson et al., 1997), a 44-bp-long control 5'-UTR (designated as syn44 5'), the *luciferase* coding region, and the *CaMV 35S* 3'-untranslated region. An almost identical construct was built, $^{35S\ R}$syn44 5'/35S 3', in which the *luciferase*$^+$ coding region was replaced by the *R. reniformis luciferase* coding region. To obtain a gene fusion construct containing both *ntp303* UTRs and the *CaMV 35S* promoter (^{35S}303 5'/303 3'), the syn44 5'-UTR was removed from ^{35S}syn44 5'/35S 3' using *Xho*I and *Nco*I restriction enzymes. The *ntp303* 5'-UTR was amplified by PCR on the *ntp303* genomic clone (Weterings et al., 1995) using the following primers with restriction sites incorporated into the 5'-end: 5'-

GTGTCTCGAGCAAGCTCTAGCAGGAAG (*Xho*I site underlined) and 5'-GTGTCCATGGGACGTTGTTTTTTTTATTC (*Nco*I site underlined). Following the PCR, the *ntp303* 5'-UTR was treated with *Xho*I and *Nco*I restriction enzymes and ligated in the ^{35S}syn44 5'/35S 3' construct (lacking the syn44 5'-UTR) to create the construct ^{35S}303 5'/35S 3'. The oligonucleotides 5'-ATATGGATCCATTCTGTAATGATCAATCTG (*Bam*HI site underlined) and 5'-ATATGAGCTCATTTAATGTTTTGTCCTA (*Sac*I site underlined) were used to generate the *ntp303* 3'-UTR using the *ntp303* genomic clone as a template. The PCR product was digested with *Bam*HI and *Sac*I and cloned into ^{35S}303 5'/35S 3' (replacing the *CaMV 35S* 3'-UTR) to create ^{35S}303 5'/303 3'.

UTR gene fusion constructs containing the *ntp303* promoter were made as follows. Using the genomic clone of *ntp303* as a template, a 578-bp-long promoter fragment, including the transcription initiation site (Weterings et al., 1995), was amplified with the primers 5'-ATATAAGCTTGATACACTCGCAACGTGTGT (*Hin*dIII site underlined) and 5'-ATATCTCGAGGAGCTTGCACTATTCACCAT (*Xho*I site underlined). The amplified *ntp303* promoter fragment was digested with *Hin*dIII and *Xho*I and, after removal of the *CaMV 35S* promoter, ligated into $^{35S\ R}$syn44 5'/35S 3', ^{35S}303 5'/35S 3' and ^{35S}303 5'/303 3' to create ^{R}syn44 5'/35S 3', 303 5'/35S 3' and 303 5'/303 3', respectively. To obtain a construct containing the *ntp303* promoter, the *ntp303* UTRs and the *R. reniformis luciferase* coding region (^{R}303 5'/303 3'), the *luciferase*$^{+}$ coding region was digested from 303 5'/303 3' using *Nco*I and *Bam*HI, after which the *R. reniformis luciferase* coding region was ligated into the *Nco*I and *Bam*HI sites. All constructs that were linked with the *ntp303* promoter and the *luciferase*$^{+}$ coding region contained a longer version of the control 5'-UTR that was used in the other constructs. This 99-bp-long control 5'-UTR was obtained by PCR using the pNBL52-42 plasmid (Bate et al., 1996) as the template. This fragment, designated as syn99 5', was amplified using the following primers: 5'-GTGTCTCGAGGATCATTGCAATTGGATCC (*Xho*I site underlined) and 5'-GTGTCCATGGCCGCGGG (*Nco*I site underlined). After removal of the *ntp303* 5'-UTR from 303 5'/35S 3' and 303 5'/303 3', the syn99 5'-UTR was cloned into the *Xho*I and *Nco*I sites, creating the syn99 5'/35S 3' and syn99 5'/303 3' constructs, respectively.

Constructs containing deletions in the *ntp303* 5'-UTR (Δ 5'-UTR) were obtained by PCR using the *ntp303* 5'-UTR in the 303 5'/35S 3' construct as starting material. In the forward primers, a *Xho*I restriction site was incorporated, whereas the reverse primers contained a *Nco*I restriction site. Schematic drawings of these modified *ntp303* 5'-UTRs are represented in Figures 7A and 8A. All fragments, which were obtained by PCR, were

sequenced completely to exclude mismatches within the sequences. All constructs used for the transient expression assays were in the pUC19 plasmid.

Microprojectile bombardment

Microcarriers, rupture discs, and macrocarriers were obtained from Bio-Rad Laboratories (Hercules, CA). Preparation and coating of the microcarriers was performed according the manufacture's manual (Bio-Rad Laboratories). For biolistic transformation of late-bicellular pollen and mature pollen, we used per bombardment 250-µg gold particles with a size of 1 and 1.6 µm, respectively. The microcarriers were coated with a total amount of 1 µg of DNA containing 0.7 µg of test construct DNA and 0.3 µg of normalization construct DNA. Test constructs containing the *ntp303* promoter and the *luc*$^+$ coding region, the *CaMV 35S* promoter, and the *luc*$^+$ coding region, or the *ntp303* promoter and the *rluc* coding region, were co-precipitated with the constructs ^{R}syn44 5'/35S 3', $^{35S\ R}$syn44 5'/35S 3', and syn44 5'/35S 3', respectively. Microprojectile bombardment was performed using the helium-driven PDS-1000/He System (Bio-Rad Laboratories). For biolistic transformation of pollen and leaves, the following bombardment parameters were used: a target distance of 6 cm, a gap distance of one-fourth inch, a macroprojectile-stopping screen distance of 8 mm, a chamber vacuum of 28 mmHg, and a burst pressure of the rupture discs of 1,100 psi.

Total RNA isolation and Northern blot analysis

To determine both the relative transcript and luciferase activity levels, pollen were separated in two fractions. One fraction was used for total RNA isolation and the other for the luciferase assay. Total RNA was isolated as described by Van Eldik et al. (1995). Ten micrograms of total RNA was denatured for 1 h at 50°C in a glyoxal/dimethyl sulfoxide mixture (Sambrook et al., 1989). After denaturation, the total RNA samples were loaded on Hybond-N$^+$ membranes according to the manufacture's manual (Amersham). The dot blots were hybridized with a ^{32}P-labeled *luciferase* probe for 20 h at 65°C in 6X SETS buffer (20X SETS stock = 3 M NaCl, 0.4% [w/v] polyvinylpyrrolidone, and 4% [w/v] bovine serum albumin), 5X Denhardts (50X stock = 1% [w/v] Ficoll, 1% [w/v] polyvinylpyrrolidone, and 1% [w/v] bovine serum albumin), 0.1% (w/v) SDS, and 75 µg/mL^{-1} denatured herring sperm DNA. Washings were performed for 30 minutes at 65°C in 2X SSC, 0.1% (w/v) SDS, 1X

SSC, 0.1% (w/v) SDS, and 0.5X SSC, 0.1% (w/v) SDS. The blots were exposed to Kodak X-omat films (Eastman-Kodak, Rochester, NY) using two intensifying screens at –80°C.

Luciferase assay

After particle bombardment and incubation of the tissues, quantitative determination of translation, as determined by the luciferase activity of the UTR gene fusion constructs, was performed using chemicals of the commercial available Dual-Luciferase Reporter Assay System (Promega). In this assay, the activities of the LUC^+ and RLUC luciferases were measured sequentially from a single sample extract using a luminometer provided with two auto-injectors (Wallac 1420 VICTOR^2, PerkinElmer, Boston). Preparation of the buffers used in the assay was performed according the manufacture's manual (Promega). After incubation, the developing pollen was transferred into a 10-mL Greiner tube and collected by centrifugation for 2 minutes at 2,500 rpm. Germinating pollen was collected by centrifugation for 5 minutes at 1,000 rpm. In all cases, the pollen pellet was resuspended in 100 µL 1X passive lysis buffer (Promega) and ground in liquid nitrogen. The pollen extracts were stored at -70°C until they were used for the luciferase activity assay. Extracts (10 µL) were pipetted in a microtiter plate, after which 100 µL of Luciferase Assay Reagent II (Promega) was added automatically. After 2 s, chemiluminescence was measured for 10 s, which gave rise to a value representing LUC^+ activity per 10-s measuring time. After quantification of the LUC^+ luminescence, the reaction was quenched and the RLUC reaction was initiated by the addition of 100 µL of Stop&Glo Reagent (Promega) to the extract. Two seconds after addition of the Stop&Glo Reagent, RLUC luminescence was measured for 10 s. This measurement represents the *R*LUC activity per 10-s measuring time. Variability of the translation between independent experiments was normalized by calculation of the ratio of LUC^+:RLUC, which gave rise to a value representing the relative luciferase activity per 10-s measuring time (RLA/10 s^{-1}).

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Chapter 3

Differential activity of the *ntp303* 5'-UTR determines temporal-related translation efficiency, and appears to be directed by processes underlying dehydration and rehydration

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ABSTRACT

Transcripts of the *ntp303* gene are produced and stored during pollen development until their utilization for translation during pollen tube growth. Previously, transient expression analysis has demonstrated the capacity of the *ntp303* 5'-UTR to enhance translation of chimeric transcripts in pollen tubes. To study the temporal activity of the *ntp303* 5'-UTR on translation in more detail throughout pollen development and pollen tube growth, stable tobacco transformants were made containing different 5'-UTR reporter gene constructs. During pollen development, the *ntp303* 5'-UTR mediated a constant translation level, whereas the translation level of chimeric transcripts was enhanced at the onset of pollen tube growth. Activity of the *ntp303* 5'-UTR gave rise to a constant protein level in pollen tubes that were growing in the style. Enhancement of translation of transcripts containing the *ntp303* 5'-UTR was also observed in rehydrated seeds. These results indicate that *ntp303* 5'-UTR activity depends on defined cellular signals that are present during the process of rehydration independently of a pollen genetic background. Furthermore, the reporter gene assay demonstrated that expression of the *ntp303* gene was not restricted to pollen, but also occurred in the female reproductive organ.

INTRODUCTION

Plant development is controlled by hierarchical networks of genes whose expression is regulated in a temporal and spatial pattern. Large-scale gene expression analyses support the idea that it is the coordinated expression rather than the number of genes that directs developmental processes. Moreover, it becomes apparent that in many developmental processes post-transcriptional rather than transcriptional regulation plays a key-role in control of gene expression. In these cases, the process of transcription and translation is often separated in time.

A well-documented example of a tissue in which expression of various genes is regulated by a successive activity of the transcription and translation machinery, is the male

gametophyte or pollen (for review, see Mascarenhas, 1990, 1993; McCormick, 1991). A pollen grain is a bi- or tricellular structure that arises from a microspore after an asymmetric cell division. The development of a pollen grain is characterized by cytological and cytochemical changes that lead to an extensive dehydration and the storage of RNAs and ribosomes. When a pollen grain lands on a compatible stigma, rehydration of the grain occurs which leads to the utilization of stored components for subsequent pollen germination and pollen tube growth.

During pollen development, at least 20,000 genes are transcribed (Willing and Mascarenhas, 1984; Willing et al., 1988) which gives rise to the appearance of several populations of mRNAs differing in their accumulation kinetics (Stinson et al., 1987; Schrauwen et al., 1990). One population is represented by transcripts that are synthesized specifically after pollen mitosis I and that accumulate in the mature pollen grain. Many of these so-called late pollen mRNAs are poorly translated during pollen development, and are stored for their utilization during pollen tube growth (for review, see Mascarenhas, 1990, 1993; McCormick, 1991; Taylor and Hepler, 1997). This phenomenon clearly emphasizes a temporal separation of transcription and translation and the presence of a coordinated fine tuning mechanism between the activity of non-coding regions that primarily determine transcription and translation, i.e. the promoter and 5'-untranslated region (5'-UTR), respectively. We hypothesized that the low or enhanced level of translation of pre-synthesized transcripts during pollen development or pollen tube growth, respectively, is reflected by a differential activity of the 5'-UTR (Hulzink et al., 2002; chapter 2 this thesis). In such a case, it may be assumed that differences in cellular conditions between developing and germinating pollen determine the differential activity of the 5'-UTR. The temporal regulation of pollen gene expression does not exclude the possibility that the promoter and 5'-UTR of late pollen genes is also active in a spatial-related manner throughout the plant. If so, we hypothesize that the coordinated activity of the pollen gene promoter and 5'-UTR differs between pollen and non-pollen tissues.

To obtain more insight in the process of delayed translation of pre-synthesized mRNAs in pollen, we concentrated on the *ntp303* gene from tobacco. Transcripts of *ntp303* accumulate during pollen development (Weterings et al., 1992), whereas the protein appears at the onset of pollen rehydration (Wittink et al., 2000). Sequences within the *ntp303* promoter direct a high level of *ntp303* gene activity exclusively in pollen (Weterings et al., 1995b), whereas sequences within the 5'-UTR enhance translation of pollen transcripts in growing pollen tubes (Hulzink et al., 2002; chapter 2 this thesis). In the present study, we

investigated the temporal effect of the *ntp303* 5'-UTR on translation of pre-synthesized mRNAs during pollen development and pollen tube growth. Therefore, stable transformants containing different promoter- and 5'-UTR combinations linked to the *luciferase*$^{+}$ (Promega, Madison, WI) coding region have been created and tested. The expression level of these reporter gene constructs was determined by measuring luciferase activity in developing pollen and growing pollen tubes in vitro and in vivo. Because the difference in water content between developing and germinating pollen is one of the most conspicuous features, we were also interested to what extent processes underlying dehydration and rehydration direct the temporal activity of the *ntp303* 5'-UTR. For this reason, we tested the activity of the *ntp303* 5'-UTR in seeds, which also exhibit successive dehydration and rehydration processes. The stable transformants enabled us also to examine to what extent *ntp303* promoter and 5'-UTR activity was restricted to the male gametophyte.

RESULTS

Expression of the pollen gene *ntp303* is not restricted to pollen, but also occurs in female reproductive tissues

The extent of *ntp303* expression throughout plant development was studied by means of a reporter gene assay in stable transformants. A combination of both a sensitive reporter gene (*luciferase*$^{+}$) and a light sensitive camera system, enabled real time monitoring of luciferase expression in the whole plant. Reporter gene constructs were made containing the *ntp303* promoter and the *ntp303* or a control 5'-UTR (Fig. 1A). For each construct, multiple independent transformants were obtained and three representative plants were selected for further analysis. Spraying of luciferin to different tissues of the transformants revealed that luciferase activity was not detectable during the vegetative growth of the plant (data not shown). Luciferin as a solution efficiently penetrates in most plant tissues after spraying (Van der Krol et al., 1999; Van Leeuwen et al., 2000). As expected, the constructs containing the *ntp303*- or control 5'-UTR revealed a strong luminescence signal in germinating pollen (Fig. 1B). Unexpectedly, a clear luciferase activity was also observed in the stigma and ovary; the activity increased upon anthesis (Fig. 1D). The luminescence signal in these tissues was

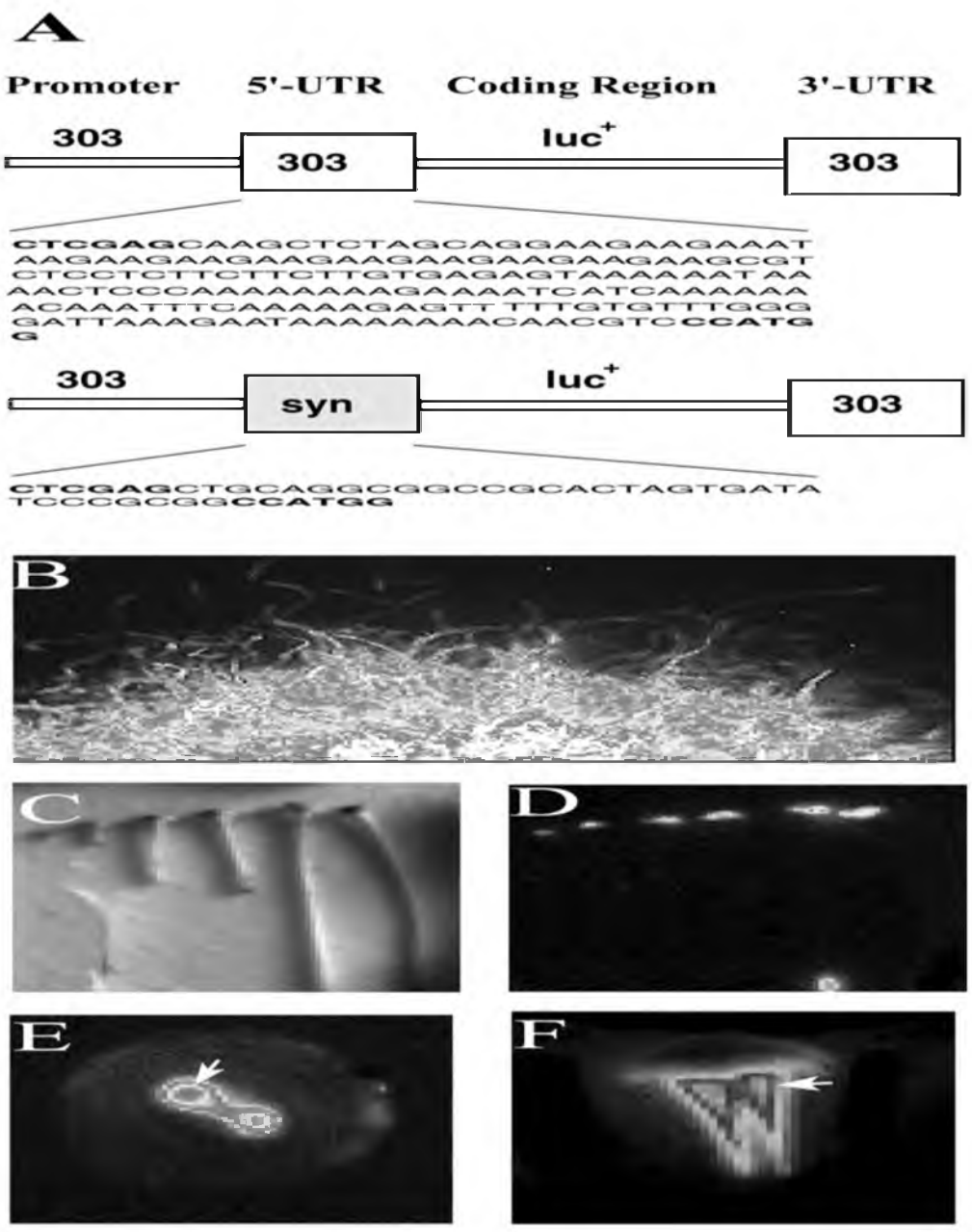

Figure 1. *Ntp303* promoter constructs and their activity in transgenic tobacco.

A, Schematic representation of the 5'-UTR reporter gene constructs containing the *ntp303* promoter, the firefly *luciferase*$^{+}$ (luc^{+}) coding region, the *ntp303* 3'-UTR, and the *ntp303* (ntp303) or control (syn) 5'-UTR. B, luminescence image of in vitro growing pollen tubes. C and D, Light and luminescence images of non-dissected transgenic tobacco pistils of different developmental phases (from 10-mm to 50-mm buds; for description of pistil development phases, see Koltunow et al. [1990]). E, Luminescence image of a transversal dissected style just under the stigma. F, Luminescence image of a longitudinal dissected pistil. Luciferase activity is represented in false gray colors. Highest luciferase activity in Figure 1E and F is indicated with a white arrow. With the exception of the pollen, all tissues are sprayed with luciferin, after which the luminescence was imaged for 15 minutes. For details about the experimental procedure, see "Material and Methods".

specific, because exclusion of luciferin gave no detectable luciferase activity. Close up analysis revealed that the luminescence signal was localized at the basis and lobes of the stigma and in the vascular bundles (Fig. 1, E and F). Each of the transformed lines showed a similar expression pattern independent of the used 5'-UTR. This indicates that expression in these tissues is primary determined by the *ntp303* promoter. *Ntp303* promoter activity in female reproductive tissues was confirmed by mRNA analysis (Fig. 2A). Although Northern

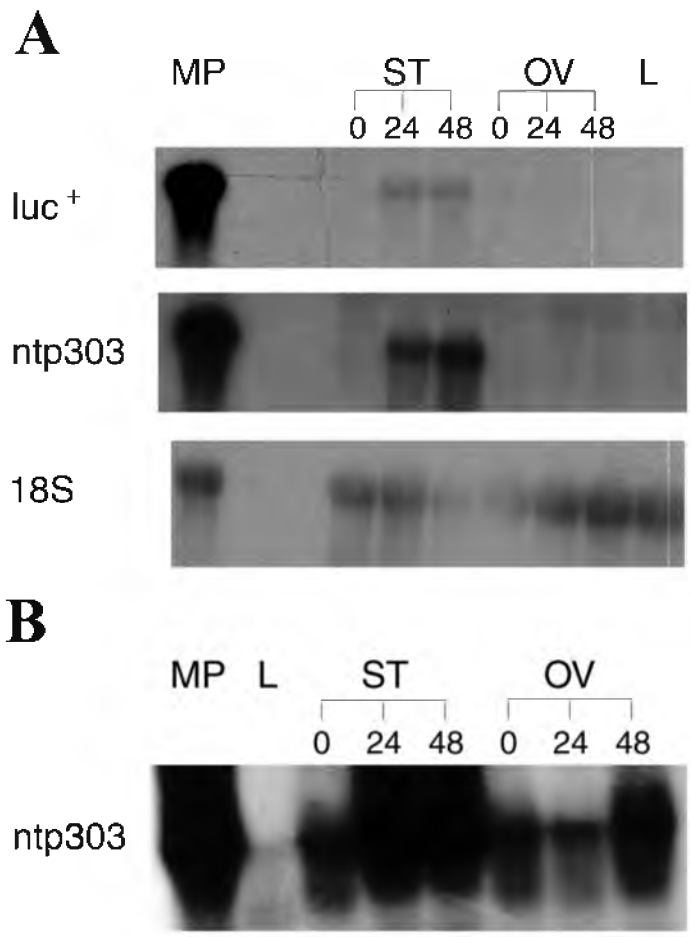

Figure 2. The presence of *luciferase*$^{+}$ and *ntp303* mRNA in the pistil of non-pollinated and pollinated wild type tobacco flowers.

A, Accumulation of *ntp303* (ntp303) and *luciferase*+ (luc+) mRNA in pistil tissues as determined with Northern analysis. The intensity of the ribosomal RNA (18S) signal is indicative for the loading of the RNA gel. B, Accumulation of *ntp303* reverse transcriptase PCR products from total RNA of pistil tissues. Abbreviations RNA lanes: MP, mature pollen; ST, style; OV, ovary; L, leave. In all cases, wild type flowers were pollinated with transgenic pollen containing the *ntp303* promoter and 5'-UTR reporter gene construct. RNA extractions were performed after 0 (0), 24 h (24), or 48 h (48) of pollination.

blot analysis was not able to proof the presence of *ntp303* mRNA in styles and ovaries of non-pollinated wild type flowers (Fig. 2A), a more sensitive RT-PCR approach using *ntp303*-specific primers revealed the presence of an *ntp303* fragment (Fig. 2B). These data evidently indicate that the endogenous *ntp303* gene is indeed active in the style and in the ovary. The strong *ntp303* mRNA signals in the pollinated styles and ovaries are predominantly due to *ntp303* mRNA from the growing pollen tubes. No *ntp303* fragments were obtained using total RNA of leaves, which is consistent with the observation that luciferase activity was absent in vegetative tissues. These results clearly demonstrate that the activity of the *ntp303* gene is not restricted to pollen, but also occurs in female reproductive tissues.

The *Ntp303* 5'-UTR enhances the translation efficiency of transcripts at the onset of pollen tube growth

The temporal effect of the *ntp303* 5'-UTR on translation throughout pollen development and pollen tube growth was studied by measuring reporter gene activity of the transformants containing the *ntp303* or the control 5'-UTR. Previously, the control 5'-UTR has been shown to mediate efficient translation of chimeric transcripts in developing pollen and pollen tubes (Bate et al., 1996; Hulzink et al., 2002; chapter 2 this thesis). From five independent transformants per construct, developing pollen and growing pollen tubes were used for total protein extraction and determination of the total protein concentration and luciferase activity (Fig. 3A). During pollen development, both 5'-UTR reporter gene constructs showed a constant luciferase activity pattern, indicating a steady translation of chimeric transcripts. However, in contrast to pollen development, the luciferase activity level mediated by the *ntp303* 5'-UTR was strongly enhanced in growing pollen tubes, whereas the control 5'-UTR gave only rise to a moderate enhancement.

In order to investigate whether the enhancement of the luciferase activity level was caused by an increase in the luciferase protein accumulation level, Western analysis was performed using an antibody against the LUC^+ protein (Fig. 3C). For both UTR reporter gene constructs, the luciferase activity pattern reflected qualitatively the respective protein accumulation pattern, indicating that enhancement of luminescence of the *ntp303* 5'-UTR construct was not due to an increase in the specific activity of the LUC^+ enzyme, but to an increase of the amount of protein.

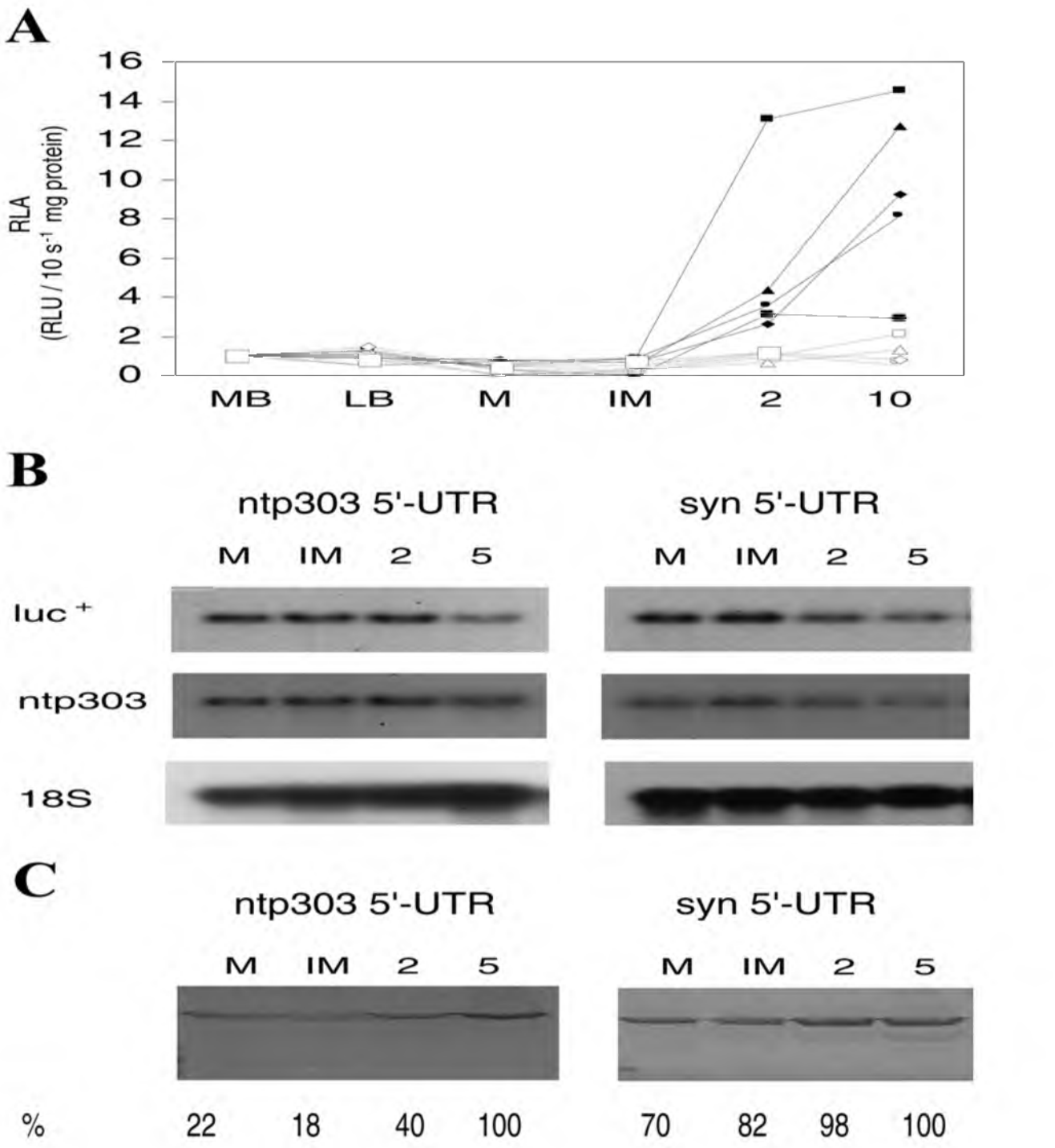

Figure 3. Analysis of luciferase expression in pollen and pollen tubes from stable tobacco transformants containing the *ntp303* or the control 5'-UTR construct.

A, luciferase activity of two reporter gene constructs during in vivo pollen development and in vitro pollen tube growth. The constructs were driven by the *ntp303* promoter and contained either the *ntp303* (black symbols) or the control (white symbols) 5'-UTR. RLA means relative luciferase activity (for details, see "Materials and Methods"). For each 5'-UTR reporter gene construct (*ntp303* 5'-UTR, left row; control 5'-UTR, right row), pollen and pollen tubes of one transformant were analyzed for *luciferase*$^{+}$ mRNA (B) and luciferase protein (C)

accumulation. The Northern blots were hybridized with either a ^{32}P-labeled *luciferase*$^{+}$ (luc^{+}), *ntp303* (ntp303), or 18S rRNA (18S) probe. The Western blots were treated with an anti-luciferase pAb antibody. The percentage underneath the Western blots represents the relative luciferase protein accumulation level in proportion to that of the 5 h germination lane (which is set on 100%). Abbreviations: M, mature; IM, imbibed pollen; 2, two h germinated pollen; 5, five h germinated pollen.

To examine whether the enhancement of translation mediated by the *ntp303* 5'-UTR was not due to an enhancement of the transcript accumulation level in the transgenic plants, Northern analysis was carried out. Total RNA was isolated from pollen of one of the transformants of each construct and the *luciferase*$^{+}$ hybridization signals were quantified after correction with the ribosomal RNA signals (Fig. 3B). Figure 3B shows that both 5'-UTR reporter gene constructs had a *luciferase*$^{+}$ accumulation pattern that represents that of the endogenous *ntp303* mRNA. The apparent absence of an increase in *luciferase*$^{+}$ mRNA accumulation during pollen tube growth clearly demonstrates that the *ntp303* 5'-UTR-mediated enhancement of luciferase activity is primarily due to an increase in translation efficiency.

In summary, the *ntp303* 5'-UTR differentially regulates translation of endogenous transcripts during the transition of developing to germinating pollen. Although the mRNA accumulation level is similar in developing and germinating pollen, the *ntp303* 5'-UTR gives rise to a high level of protein synthesis at the onset of pollen germination after its stable integration into the tobacco genome. In this respect, it is apparent that cellular conditions of growing pollen tubes direct the high activity of the *ntp303* 5'-UTR.

The *ntp303* 5'-UTR maintains a constant translation activity during in vivo pollen tube growth

To follow the high activity of the *ntp303* 5'-UTR during pollen tube growth in the style and ovary, the luciferase activity level of the reporter gene constructs containing the *ntp303* and control 5'-UTR was determined at different time points of in vivo pollen tube growth (Fig. 4A). Transgenic pollen from three independent transformants per construct was put on wild type stigmas and the luciferase activity was measured in total protein extracts of the pollinated pistils. After 24 h of in vivo pollen tube growth, the *ntp303* 5'-UTR gave rise to a luciferase activity level that was almost 3-fold higher than that of the control 5'-UTR construct. During subsequent pollen tube growth (48 and 72 h after pollination), the luciferase

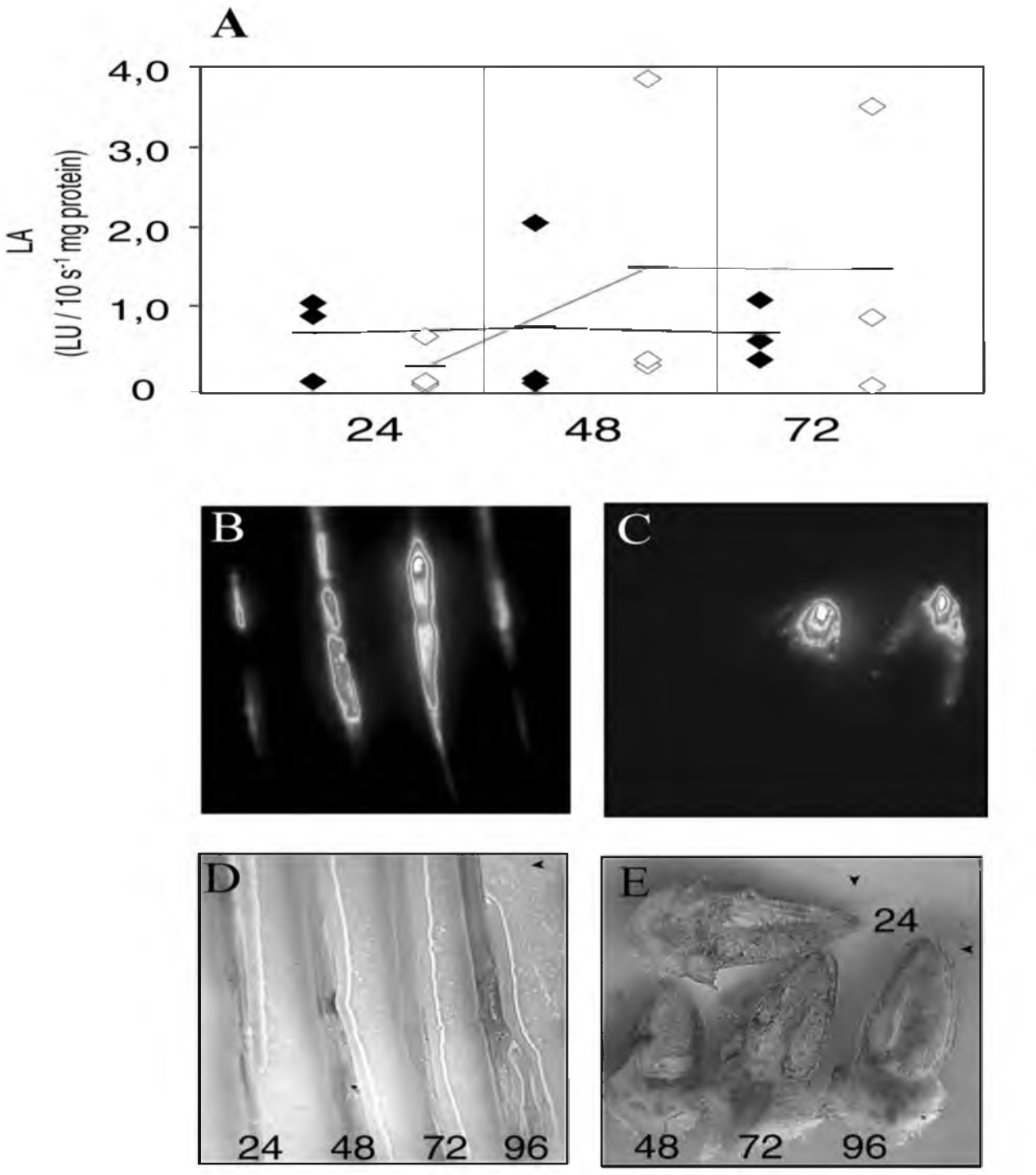

Figure 4. Real time luciferase activity of 5'-UTR reporter gene constructs in transgenic pollen tubes within wild type tobacco pistils.

A, Luciferase activity of reporter gene constructs containing the *ntp303* (black symbols) or control (white symbols) 5'-UTR in vivo growing pollen tubes within wild type pistils. Each symbol represents the activity value from pollen of one independent transformant, which has been pollinated on two wild type flowers (for details, see "Materials and Methods"). LA means luciferase activity. D and E, Light images of longitudinal dissected styles (D) and ovaries (E) of wild-type tobacco flowers pollinated with transgenic pollen (containing

the *ntp303* promoter and 5'-UTR construct). The upper part of the styles and ovaries are indicated with arrowheads. B and C, Luminescence images of the dissected styles (B) and ovaries (C) of the light images. Luciferase activity is represented in false gray colors. Transgenic pollen was imbibed in a germination medium containing luciferin. In all cases, the wild type flowers were pollinated with transgenic pollen and luciferase activity was monitored after 24, 48, 72, and 96 h of in vivo pollen tube growth.

activity level of the *ntp303* 5'-UTR construct exhibited a steady state pattern, whereas the control 5'-UTR construct gave rise to an increase in the luciferase activity level. With regard to the in vivo half-life of the luciferase protein (2.5 h; Van Leeuwen et al., 2000), it is apparent that the measured luminescence at the different time points originated from luciferase enzymes that were synthesized to a maximum of five h before. In this respect, it is apparent that the *ntp303* 5'-UTR is active throughout pollen tube growth. This is confirmed by a qualitative in vivo luciferase detection experiment using a 2D-luminometer (Fig. 4, B and C). Actual changes in *luciferase*$^+$ gene expression can be measured due to biochemical characteristics of the luciferase enzyme. Addition of luciferin to luciferase causes the formation of a stable luciferase/oxyluciferin complex that leads to the exclusion of the enzyme from new catalytic activities (DeLuca and McElroy, 1974; Aflalo, 1991). Figure 4C clearly demonstrates that dissected and luciferin-treated styles of wild type tobacco flowers that were pollinated with transgenic pollen containing the *ntp303* 5'-UTR construct showed a strong luminescence signal throughout pollen tube growth. Real time luciferase activity was also observed in ovaries at 72 and 96 h after pollination (Fig. 4E). These observations unmistakable demonstrate that the *ntp303* 5'-UTR is highly active in pollen tubes from pollination to the time point of fertilization. Additional support for the prolonged activity of the *ntp303* 5'-UTR is the presence of pollen tube *luciferase*$^+$ mRNA in wild type styles pollinated for different hours with transgenic pollen (Fig. 2A).

Activity of the *ntp303* 5'-UTR is modulated by processes of dehydration and rehydration independent from a pollen-specific environment

It is obvious that the differential effect of the *ntp303* 5'-UTR on translation in the male gametophyte is correlated with differences between cellular conditions of developing and germinating pollen. The final phase of pollen development is characterized by progressive dehydration of the pollen grain, whereas extensive rehydration occurs at the onset of pollen germination. To test whether the *ntp303* 5'-UTR had a differential effect on translation in a

non-pollen tissue that undergoes similar successive dehydration and rehydration processes, luciferase activity of the *ntp303* and control 5'-UTR constructs was measured in dehydrated and rehydrated transgenic tobacco seeds (Fig. 5).

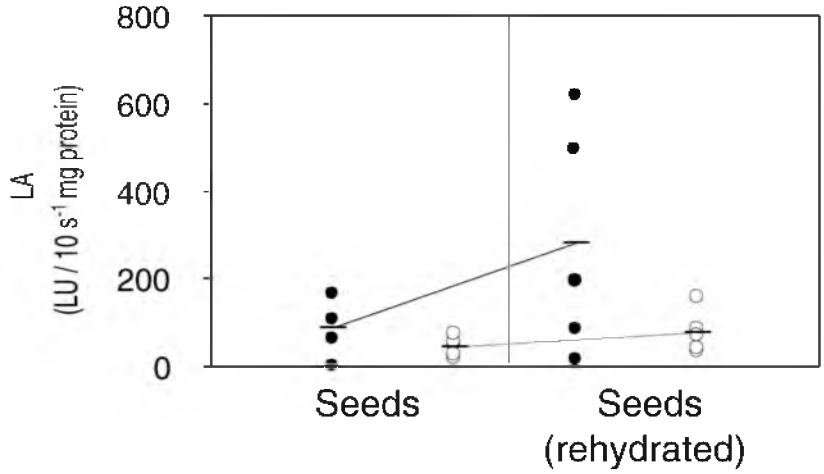

Figure 5. Luciferase activity of gene fusion constructs containing the *CaMV 35S* promoter and the *ntp303* (black dots) or control (white dots) 5'-UTR in dehydrated and rehydrated tobacco seeds.

Each dot represents the value from one independent transformant. For each independent transformant, an equal amount of seeds has been used for protein extraction. Levels of luciferase activity (LA) were determined by measuring the amount of luciferase units per 10-s measuring time in 10 mg total protein (LU / 10 s^{-1} mg protein).

Because the *ntp303* promoter constructs gave no luciferase expression in seeds (data not shown), transcription of the reporter gene constructs had to be driven by the *cauliflower mosaic virus* (*CaMV*) *35S* promoter. Luciferase activity was measured from total protein extracts of five independent transformants per construct. In dehydrated seeds, the luciferase activity level of the *ntp303* 5'-UTR construct was slightly higher than that of the control 5'-UTR construct. However, in rehydrated seeds, the *ntp303* 5'-UTR increased the activity of luciferase to a level that was almost 4-fold higher than that of the control 5'-UTR construct. These data indicate that the differential activity of the *ntp303* 5'-UTR is determined by processes of dehydration and rehydration.

DISCUSSION

In the present study, the activity of the *ntp303* 5'-UTR has been investigated during in vivo pollen development and pollen tube growth by means of a reporter gene approach in stable transformed tobacco plants. Furthermore, activity of the *ntp303* promoter and 5'-UTR has been studied in non-pollen tissues. The stable transformants showed that *ntp303* gene activity is not restricted to pollen, but also occurs in female reproductive tissues. Treatment of non-destructed transgenic pistils with luciferin revealed a high level of luciferase expression in the stigma and ovary (Fig. 1D). More specifically, *ntp303* gene activity was observed in the basis and the lobes of the stigma and in the vascular bundles (Fig. 1, E and F). Because the expression was absent in the young pistil and increased in pistils upon anthesis, *ntp303* gene activity seems to be related to the development phase of the flower. The luciferase activity in the female reproductive tissues is in agreement with the presence of *ntp303* transcripts in non-pollinated pistils (Fig. 2B). Since no differences were observed in the luciferase activity between constructs containing the *ntp303* or control 5'-UTR, the extent of *ntp303* gene expression in female reproductive tissues seems to be primary determined by the *ntp303* promoter. A strong luminescence signal was also observed in non-destructed anthers, which is mostly due to developing pollen (data not shown). However, preliminary in situ mRNA hybridization experiments demonstrated the presence of *ntp303* mRNA in the vascular bundle of anthers (data not shown), indicating that *ntp303* is also expressed in sporophytic tissues of the male reproductive organ.

Analysis of reporter gene expression during male gametogenesis reveals that the *ntp303* 5'-UTR is an important determinant of the translatability of endogenous transcripts during pollen development and pollen tube growth. When stably integrated into the tobacco genome, the *ntp303* 5'-UTR mediates a constant level of translation of transcripts during pollen development, but enhances their translation specifically at the onset of pollen tube growth (Fig. 3A). The strong enhancement effect is due to the activity of the *ntp303* 5'-UTR, because the control 5'-UTR gave only rise to a slight increase in the luciferase activity level. The *ntp303* 5'-UTR-mediated enhancement of translation is regulated at the post-transcriptional level, since enhancement of the luciferase activity and protein accumulation levels is not reflected by a similar increase of the *luciferase*$^{+}$ transcript accumulation level. These observations are in accordance with previous data from transient expression analysis

(Hulzink et al., 2002; chapter 2 this thesis). Activity of the *ntp303* 5'-UTR during pollen tube growth in the style and ovary (Fig. 4, B and C) resulted in a constant level of translation (Fig. 4A). Several observations support prolonged *ntp303* expression throughout pollen tube growth, like the durable presence of *ntp303* transcripts in growing pollen tubes (Fig. 2, A and B; Weterings et al., 1995a) and the high stability of *ntp303* transcripts (Ylstra and McCormick, 1999). In contrast to that of the *ntp303* 5'-UTR construct, the luciferase activity level of the control 5'-UTR construct increased during late phases of pollen tube growth. Since this increase is reflected by an increase of the respective *luciferase*$^{+}$ mRNA accumulation level (data not shown), we assume that this is due to de novo translation of newly synthesized and accumulating transcripts.

In plants, several examples exist of the capacity of the 5'-UTR to direct translation during development (Bailey-Serres and Dawe, 1996; Bate et al., 1996; Dickey et al., 1998; Anderson et al., 1999; Bailey-Serres, 1999; Hua et al., 2001; Paradkar and Marcotte, 2001; Bonaventure and Ohlrogge, 2002). In these cases, translation efficiency often has been demonstrated to be regulated at the post-transcriptional level. The role of the 5'-UTR in regulating translation efficiency fits the scanning model of protein synthesis in which the pre-initiation complex moves over the 5'-UTR to seek for the translation initiation codon (Kozak, 1999).

With regard to cellular features of mature pollen and pollen tubes, it is most likely that the difference in translatability of *ntp303* transcripts before and after anthesis is related to processes that underlie the successive dehydration and rehydration of pollen grains. This assumption is strengthened by the observation that the stimulatory effect of the *ntp303* 5'-UTR on translation occurs also in rehydrating seeds (Fig. 5). Although the *ntp303* 5'-UTR-mediated enhancement effect is highest in germinating pollen, these results conclusively demonstrate that it is rather the process of rehydration than the genetic background of pollen that determines the enhancement activity of the *ntp303* 5'-UTR. During pollen development, regulatory elements within the *ntp303* promoter mediate a high level of *ntp303* mRNA synthesis (Weterings et al., 1992; Weterings et al., 1995b). Consequently, these transcripts are preserved throughout pollen development for their utilization during subsequent pollen tube growth. Since both the *ntp303* and control 5'-UTR reporter gene constructs showed a transcript accumulation level which is comparable with that of endogenous *ntp303* transcripts (Fig. 3B), we assume that preservation of transcripts during pollen development does not require specific sequences within the 5'-UTR, but is mediated by processes underlying dehydration of the pollen. With regard to this, it is assumable that the 5'-UTR sequences that

have been shown previously to mediate *ntp303* mRNA stability (Hulzink et al., 2002, chapter 2 this thesis) play an important role in particular during late phases of pollen tube growth.

The present study conclusively demonstrate that the *ntp303* 5'-UTR mediates translation of *ntp303* transcripts during pollen development, but enhances translation specifically at the onset of pollen tube growth. During late phases of pollen tube growth in the style, the *ntp303* 5'-UTR mediates translation in such way that a constant protein level is guaranteed. It is apparent that the temporal activity of the *ntp303* 5'-UTR is directed by defined cellular conditions of pollen and pollen tubes through interaction of specific factors with sequences in the *ntp303* 5'-UTR. Previously, we have demonstrated that sequences within the *ntp303* 5'-UTR are important for modulation of translation efficiency of chimeric *ntp303* transcripts (Hulzink et al., 2002; chapter 2 this thesis). With regard to the occurrence of the enhancement effect in both rehydrated pollen and seeds, we assume that autonomous cellular signals derived from dehydration and rehydration processes modulate the differential activity of the *ntp303* 5'-UTR. In this respect, it is would be interesting to examine which cellular signals in pollen and seed are responsible for modulation of *ntp303* 5'-UTR activity. A first attempt has been made to identify these signals or factors in pollen by means of phage display.

MATERIALS AND MEHODS

5'-UTR reporter gene constructs and plant transformation

With the exception of one, all 5'-UTR reporter gene constructs have been described elsewhere (Hulzink et al., 2002; chapter 2 this thesis). These construct have previously been named 303 5'/303 3', syn44 5'/303 3', and ^{35S}303 5'/303 3'. The constructs contain either the *ntp303* (578 base pairs fragment) or the *CaMV 35S* promoter. All constructs contain a modified version of the firefly luciferase coding region (*luciferase*$^{+}$) and the *ntp303* 3'-UTR. The control 5'-UTR (previously labeled syn44 5') is a 44-bp-long sequence that mediates efficient translation of chimeric luciferase transcripts during pollen development and pollen tube growth (Bate et al., 1996; Hulzink et al., 2002; chapter 2 this thesis). The new construct contains the *CaMV 35* promoter, the control 5'-UTR (syn44 5'), the *luciferase*$^{+}$ coding region, and the *ntp303* 3'-UTR. To obtain this construct, the *ntp303* 5'-UTR was removed

from ^{35S}303 5'/303 3' using *Xho*I and *Nco*I restriction enzymes. The control 5'-UTR was obtained by its digestion (*Xho*I and *Nco*I) from the syn44 5'/303 3' construct. The control 5'-UTR fragment was ligated in ^{35S}303 5'/303 3' (lacking the *ntp303* 5'-UTR).

For stable transformations, the UTR reporter gene constructs (promoter, 5'-UTR, *luciferase*$^{+}$ coding region, and 3'-UTR fragment) were ligated in the *Hin*dIII and *Sac*I sites of the binary vector pBin19. The obtained constructs were transferred directly into *Agrobacterium tumefaciens* strain LBA4404 (pAL4404) according to Hofgen et al. (1988). Transformation was performed in leaf discs of *Nicotiana tabacum* (tobacco) L. cv. Petit Havana SR1 (Horsch et al., 1985). To induce shoot formation, the leaf discs were cultivated on MS30 medium containing 0.5% (w/v) kanamycin, 2% (w/v) cefotaxim, and 0,01% (w/v) 6-benzylaminopurine. Selection and induction of root formation was performed by transferring the shoots to MS30 medium containing kanamycin (0.5% [w/v]) and cefotaxim (2% [w/v]). After root formation, the shoots were transferred to soil and grown to maturity in the greenhouse. Kanamycin-resistant plants from the T2 or later generations containing one tDNA insertion were used for subsequent expression analyses.

RNA analysis and RT-PCR

Total RNA isolation and Northern blotting were performed as described earlier (Hulzink et al., 2002; chapter 2 this thesis). RT-PCR was performed using the commercial available Access RT-PCR System kit (Promega). Primers that were used for the identification of *ntp303* transcripts were designed on the 5'- and 3'-part of the coding region of *ntp303*. The sequences of the primers are: 5'- TTGCTACTTTGCCTCTCCGTAGG (32 nucleotides upstream of the translation initiation site) and 5'- GTCCATGCCAGCTCCATACAAGG (27 nucleotides upstream of the translation termination site). For each RNA sample, exclusion of reverse transcriptase to the reaction mixtures was used as a control reaction. Blotting of the obtained RT-PCR fragments was done on Hybond-N^{+} membranes according the manufacture's manual (Amersham, Buckinghamshire, UK). Hybridization conditions were equally to those of the Northern blots and are described elsewhere (Hulzink et al., 2002; chapter 2 this thesis).

Protein isolation and Western analysis

For Western analysis, total protein extracts from pollen were obtained after homogenization in 50 mM Tris-HCl (pH 6.8), 10 % (w/v) sucrose and 1 % (v/v) ß-mercaptoethanol. The membrane and wall fraction was removed by centrifugation of the extract for 5 min at 14,000 rpm in an Eppendorf centrifuge (5414C). The Bradford reagent assay (Bio-Rad Laboratories, Hercules, CA) was used for the determination of the concentration of total protein in the fractions (according the manufacture's instructions). Per sample, 50 µg total protein was used for protein electrophoresis and Western blotting (for procedure, see Wittink et al., 2000). Luciferase protein detection was performed using the commercial available primary antibody anti-luciferase pAb (Promega) at a 1:1,000 dilution along with a rabbit anti-goat IgG AP conjugate (Pierce, Rockford, IL) at a 1:5,000 dilution. The blots were developed with 0.33 mg/mL nitro blue tetrazolium (NBT) and 0.165 mg mL^{-1} 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in alkaline phosphate buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$).

Protein isolation and in vitro luciferase measurement

Pollen of different developmental phases was isolated from flower buds as described elsewhere (Tupý et al., 1991; Van Herpen et al., 1992). Seeds were rehydrated for 1 h in a Petri dish containing two layers of moistened filter paper. For pollination experiments of transgenic pollen on wild type stigmas, much care was taken to ensure that an equal amount of pollen was pollinated on each flower. Per transformant, isolated mature pollen was suspended in M1 medium (Tupý et al., 1991) at a density of 10^8 cells mL^{-1}, after which 10 µl of the suspension was pipetted onto two wild type stigmas. After pollen tube growth, the pistils were used for one protein extraction (per independent transformant). Total protein extracts were obtained after homogenization of frozen tissue in 25 mM Tris-HCl (pH 7.8), 2 mM 1,2-diaminocyclohexane-N, N, N', N'-tetra-acetic acid, 2 mM dithiothreitol, 10% (v/v) glycerol and 1% (v/v) Triton X-100. Insoluble cell material was removed by centrifugation of the extract for 5 min at 14,000 rpm in an Eppendorf centrifuge (5414C). The protein extracts were stored at -70°C until they were used for concentration and luciferase activity measurements. The concentration of total protein was determined using Bradford reagent according the manufacture manual (Bio-Rad Laboratories).

Chemiluminescence of 10 μL protein extract was measured in a luminometer containing an auto-injector (Wallac 1420 VICTOR^2, Perkin-Elmer, Boston). Hundred microliters luciferase assay buffer (5 mM ATP, 25 mM HEPES-KOH pH 7.8, 10 mM $MgCl_2$, 0.5 mM *D*-luciferin) was added automatically to the samples and the chemiluminescence was measured for 10 s (after a 2-s delay). This measurement gave rise to a value representing the luciferase activity (luciferase units; LU) per 10-s measuring time. Normalization of the luciferase activity was done by calculation of the luciferase activity per mg of total protein. This value represents the luciferase activity (LA = LU/10 s^{-1} mg total protein). RLA represents the relative luciferase activity. The mid-bicellular pollen (MB) LA values of each transformant were set to one, after which the LA values of the other pollen phases were proportional normalized.

In situ luciferase activity measurement

Dissected or non-dissected tissues of transgenic tobacco plants were sprayed with a luciferin solution containing 1 mM firefly *D*-luciferin and 0.01 % (v/v) Tween80. The activity of the luciferase enzyme in plant tissues was imaged with a 2D-luminometer containing an intensified CCD camera (C2400-77, Hamamatsu Photonics). The emission of the photons was processed by computer (Argus-50 Image processor, Hamamatsu Photonics). This equipment was also used for the detection of real time luciferase activity in vitro germinating pollen. Imbibed pollen was germinated in Read germination medium (Read et al., 1993a; 1993b) containing 0.5 mM firefly *D*-luciferin.

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Chapter 4

Structural and functional conservation of 5'-UTR sequences of homologous pollen-expressed genes from *Petunia hybrida*, *Nicotiana tabacum*, and *Lycopersicon esculentum*

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ABSTRACT

In order to investigate to what extent genetic programs that determine delayed translation of stored pollen mRNAs are conserved at the molecular level in closely related plant species, a comparative study of three homologous pollen-expressed genes from petunia (*php303*), tobacco (*ntp303*), and tomato (*lat51*) has been performed. Transcripts of the *ntp303* gene are stored in developing pollen, until their utilization during pollen tube growth. Comparison of the *php303* mRNA and protein accumulation patterns in petunia pollen revealed also a delayed of *php303* transcripts during pollen development. Transient expression of different 5'-untranslated region (5'-UTR) reporter gene constructs demonstrated the capacity of the *php303* 5'-UTR to enhance gene expression in pollen tubes. Alignment of the 5'-UTR of *php303*, *ntp303* and *lat51* revealed a high level of sequence similarity, especially in sequence regions that have been shown earlier to determine translation efficiency of *ntp303* transcripts in tobacco pollen tubes. These results indicate structural and functional conservation of 5'-UTR sequences in homologous pollen-expressed genes from different plant species.

INTRODUCTION

Development of the male gametophyte (pollen) requires a coordinated expression of genes in both gametophytic and in sporophytic tissues (for review, see Mascarenhas, 1989; Scott et al., 1991; Goldberg et al., 1993; McCormick, 1993; Schrauwen et al., 1996). To fulfill its final biological function, the delivery of the sperm cells to the ovules, pollen undergoes complex developmental programs that are highly conserved between mono- and dicotyledonous plant species. On basis of the accumulation patterns of mRNAs, pollen-expressed genes from various plant species can be divided in several populations (Stinson et al., 1987; Schrauwen et al., 1990). Transcripts of the so-called late pollen genes are synthesized throughout pollen development and exhibit a maximum accumulation level in mature pollen. Many of these late pollen mRNAs are stored during pollen development, until their utilization at the onset of pollen tube growth (Schrauwen et al., 1990; Mascarenhas,

1993; Štorchová et al., 1994). Initial protein synthesis at the onset of pollen germination mainly depends on the utilization of stored mRNAs (Čapková et al., 1988; Mascarenhas, 1993; Štorchová et al., 1994). Here, post-transcriptional control of gene expression is very essential for successful fertilization. An example of a late pollen gene which exhibits delayed translation of its mRNA at the onset of pollen tube growth is *ntp303* from tobacco (Weterings et al., 1992; Čapková et al., 1994; Štorchová et al., 1994; Wittink et al., 2000).

Despite the importance of post-transcriptional processes for regulation of pollen gene expression, relative little attention has been paid to identify and characterize regulatory sequences that are important for directing delayed translation of stored pollen transcripts (Dawe et al., 1993; Bate et al., 1996; Curie and McCormick, 1997; Hulzink et al., 2002; chapter 2 and 3 this thesis). Recently, sequence regions within the 5'-untranslated region (5'-UTR) of *ntp303* have been demonstrated to determine stability and translation efficiency of transcripts during the transition of mature to germinating pollen (Hulzink et al., 2002; chapter 2 this thesis). The widespread occurrence of post-transcriptional regulation of translation of pollen mRNAs in various plant species led to the hypothesis that components of the genetic programs that determine the temporal-related translation efficiency of pollen mRNAs might be evolutionary preserved. If so, it may be assumed that the 5'-UTR of transcripts that exhibit delayed translation during male gametogenesis comprises analogous regulatory sequences that are involved in post-transcriptional regulation of translation. In this respect, a comparative study of homologous genes from closely related species is an excellent opportunity to test this hypothesis. Therefore, we aimed to investigate to what extent the *ntp303* 5'-UTR is conserved in homologous genes from petunia (*Petunia hybrida*) and tomato (*Lycopersicon esculentum*). The petunia homologue *php303* was isolated as a genomic clone and its sequence and expression characteristics were determined. Subsequently, the 5'-UTR sequences of *ntp303* and *php303* were compared with that of the tomato homologue *lat51*.

RESULTS

Php303, a petunia homologue of the tobacco pollen gene *ntp303*

A PCR-based strategy was used to isolate a petunia homologue of the pollen-expressed gene *ntp303* from tobacco. Annotation of the obtained amplification products

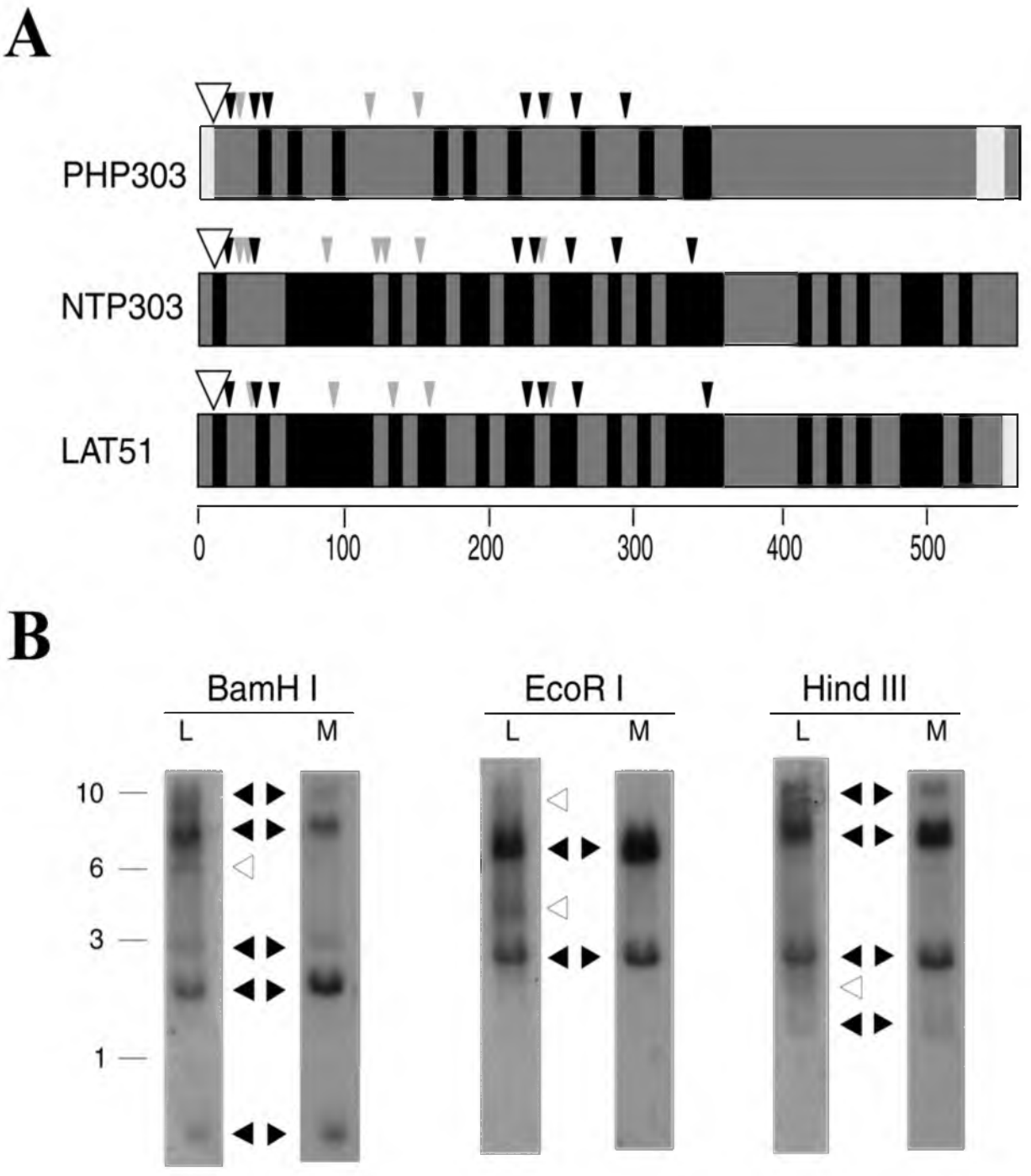

Figure 1. Schematic representation of the deduced PHP303, NTP303, and LAT51 proteins (A) and genomic organization of the *php303* gene (B).

A, The degree of sequence similarity between the proteins is indicated. Black, dark gray, and light gray means a complete, high, and low level of amino acids identity, respectively. The small arrows indicate the positions of predicted glycosylation (black) or myristoylation (gray) sites. The large white arrows indicate the position of predicted signal peptide cleavage sites. The numbers indicate position of amino acids. B, Hybridization of a ^{32}P-labelled *php303* coding region probe with digested petunia genomic DNA after low (L) or moderate (M) stringent washings (for details procedure, see "Materials and Methods"). Numbers at the left represent the sizes (kb) of the genomic DNA fragments.

resulted in a 2222-bp-long genomic DNA fragment, *php303* (named *p303* in GenBank database; accession number AF479568). Comparison of both the complete nucleotide and the deduced protein sequence of *php303* with sequences in the GenBank database (Fig. 1A) revealed a high homology to *ntp303* from tobacco (80% nucleotide sequence identity) and *lat51* from tomato (92% nucleotide sequence identity).

Southern analysis was performed to determine the number of *php303*-related genes in the petunia genome. DNA was isolated from leaves of petunia and digested with BamHI, EcoRI and HindIII (Fig. 1B). The *php303* gene contains a BamHI and a HindIII site in its nucleotide sequence. Figure 1B shows the hybridization signals of a ^{32}P-labelled *php303* probe at low and moderate stringencies. Under moderate stringent washing conditions, the BamHI and HindIII lanes revealed the presence of at least two strong and two weaker hybridizing fragments. The EcoRI lane showed the presence of two strong hybridizing fragments. With regard to the absence or presence of the EcoRI or BamHI and HindIII sites, respectively, the Southern blot results indicate the presence of at least two *php303* copies in the petunia genome. Low stringency washings revealed the appearance of additional weak hybridizing fragments in all lanes. These fragments probably represent genes that exhibit low homology to *php303*.

The accumulation pattern of *php303* mRNA is similar to that of *ntp303* transcripts

To investigate the extent of *php303* mRNA accumulation in various tissues of petunia, Northern analysis was carried out (Fig. 2, A and B). Using a ^{32}P-labelled *php303* probe, no hybridization signal was detected in the total RNA lanes of different sporophytic tissues (Fig. 2A). In the total RNA lane of mature petunia pollen, a strong hybridization signal was observed to a transcript of approximately 2000 nucleotides in length. This size corresponded with the predicted transcript length of *php303*. The *php303* probe hybridized to a transcript of approximately 2100 nucleotides in the total RNA lane of mature pollen from tobacco. It is obvious that this cross-hybridization occurred to transcripts of the tobacco *ntp303* gene. During pollen development, *php303* transcripts were already detectable in early-bicellular pollen (Fig. 2B). From this stage on, the amount of *php303* transcripts continued to increase until the mature pollen phase. During subsequent pollen germination, a decrease in the *php303* mRNA accumulation level was observed. The Northern blot data clearly demonstrate

that the *php303* mRNA accumulation pattern in pollen is very similar to that of the *ntp303* gene.

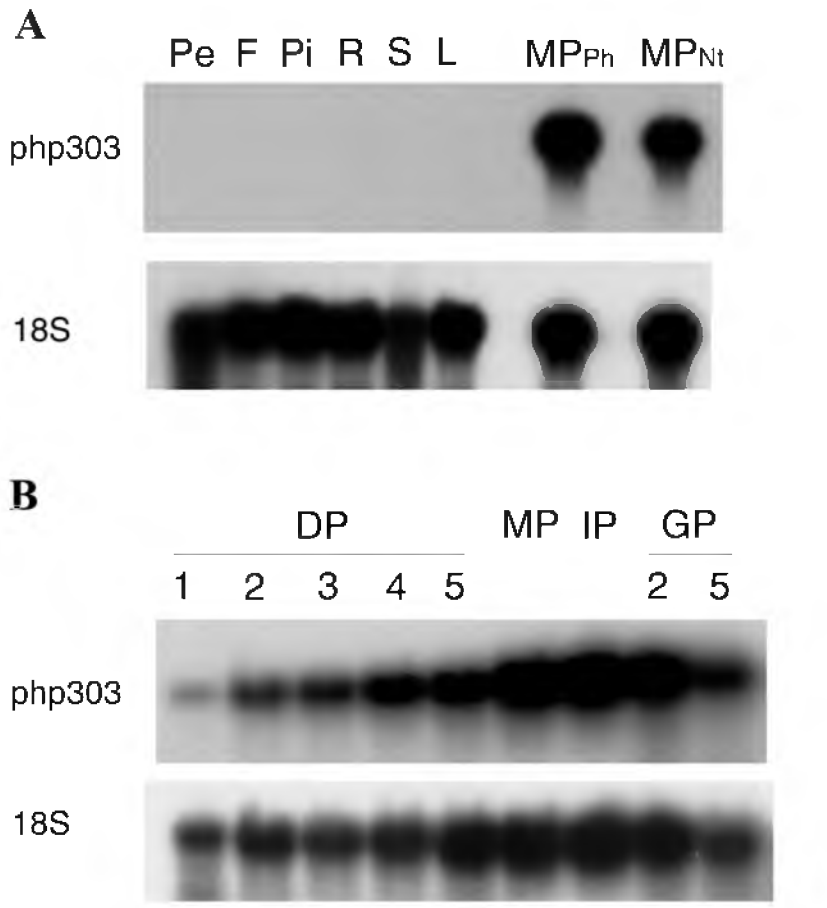

Figure 2. Analysis of the accumulation of *php303* mRNA in different tissues (A) and during pollen development and pollen tube growth (B).

Each lane contains ten microgram total RNA. The Northern blots are hybridized with a ^{32}P-labeled *php303* coding region (php303) or a ribosomal 18S (18S) probe. Abbreviations tissues: Pe, petal; F, filament; Pi, pistil; R, root; S, stem; L, leaf; MPph, mature pollen *Petunia hybrida*; MPnt, mature pollen *Nicotiana tabacum*. Abbreviations pollen: DP, developing pollen from early- (1) to late- (5) bicellular phase; MP, mature pollen; IP, imbibed pollen; GP, germinating pollen (2 and 5 h).

Pph303 mRNA is translated during pollen development

Western analysis was performed to determine whether the course of PHP303 protein accumulation during pollen development and pollen tube growth was similar to that of the NTP303 protein in tobacco. The presence of PHP303 in total protein fractions was determined using an antibody that was raised against the carboxyl-terminal part of the tobacco NTP303

protein. With regard to the high level of sequence similarity between the PHP303 and NTP303 proteins, we expected a cross-reaction of the PHP303 protein with the C-terminal-based antibody of NTP303. Figure 3 shows an immunoblot containing total protein of soluble (cytoplasmic) extracts of petunia pollen from different developmental phases.

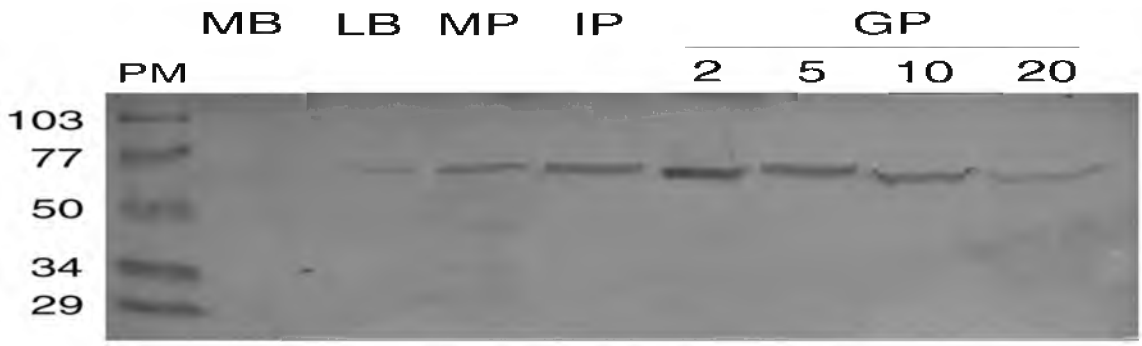

Figure 3. Analysis of the accumulation of the PHP303 protein in the cytosol and membrane total protein fractions from developing pollen and growing pollen tubes.

Each lane contains fifty micrograms total protein. PHP303 protein detection was performed using a NTP303 C-terminal specific antibody along with a goat anti-rabbit AP conjugate. The Western blots were treated with NBT and BCIP to visualize the proteins. Abbreviations pollen: MB, mid-bicellular pollen; LB, late-bicellular pollen; MP, mature pollen; IP, imbibed pollen; GP, germinating pollen (2 to 20 h). Numbers at the left indicate the molecular weight (kDa) of the proteins as determined by a protein molecular weight marker (PM).

In the cytoplasmic protein fractions, the presence of a 60 to 70-kDa protein was detectable in late-bicellular pollen. From this phase on, the amount of protein increased during subsequent pollen development and pollen tube growth. Unlike the NTP303 protein, the PHP303 protein was not detectable in the insoluble or cell wall fractions of developing and germinating petunia pollen (data not shown). With regard to the time point of appearance, the PHP303 protein differs from that of NTP303. The PHP303 protein was already detectable in developing petunia pollen, whereas the 69-kDa NTP303 protein starts to accumulate at the onset of tobacco pollen germination (Wittink et al., 2000). With regard to the abundant presence of *php303* mRNA in early-bicellular pollen, the appearance of the PHP303 protein from the late-bicellular pollen phase clearly indicates a delayed translation of *php303* transcripts during pollen development.

The 5'-UTR of *php303* enhances translation in pollen

Previous analysis has demonstrated that the 5'-UTR of the *ntp303* gene is an essential determinant for the high level of NTP303 synthesis at the onset of pollen tube growth (Hulzink et al., 2002; chapter 2 and 3 this thesis). In order to investigate whether the *php303* 5'-UTR was also able to enhance translation in petunia pollen, transcripts of four 5'-UTR gene fusion constructs were tested for their translatability by means of a transient expression assay (Fig. 4, A and B).

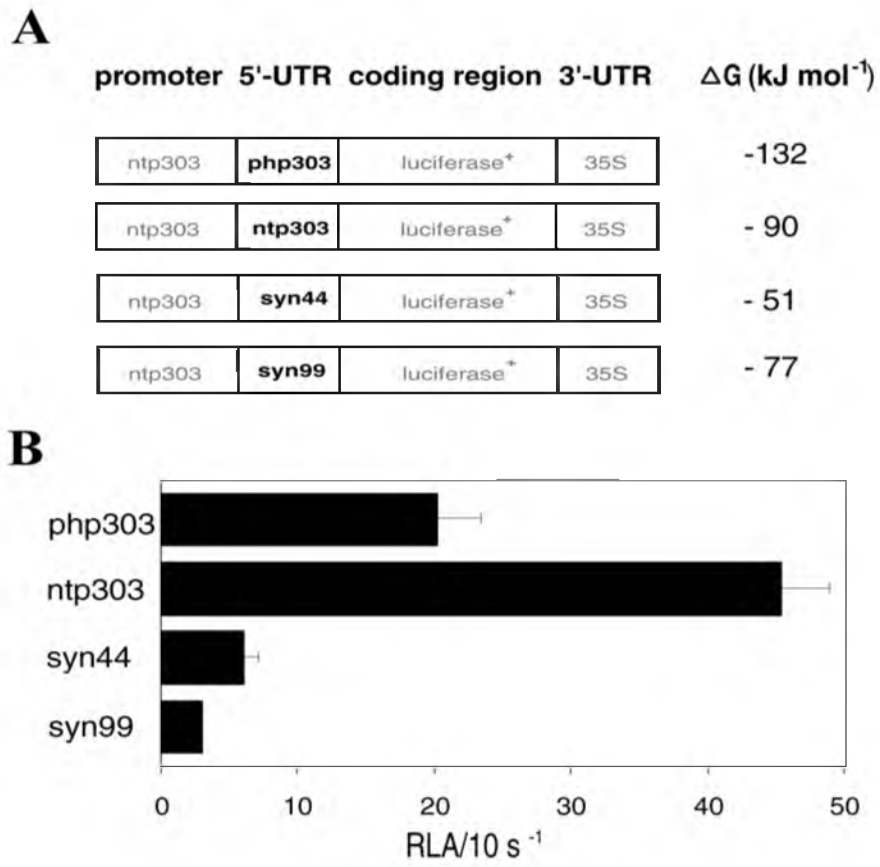

Figure 4. 5'-UTR reporter gene constructs (A) and their transient expression in petunia pollen tubes (B).

A, Schematic representation of the 5'-UTR reporter gene constructs containing the *ntp303* promoter, the firefly *luciferase*$^{+}$ coding region, the *CaMV 35S* termination sequence, and different 5'-UTRs. The calculated secondary structure stability (ΔG) of each 5'-UTR is given in the right column. B, Luciferase activity of the 5'-UTR reporter gene constructs in petunia pollen tubes after 20 h of growth. RLA/10 sec^{-1} means the relative luciferase activity (luminescence) per 10-s measuring time after normalization with the luciferase activity of the reference construct syn44 5'/35S 3' (*ntp303* promoter, syn44 5'-UTR, *CaMV 35S* termination sequence, and *Renilla reniformis luciferase* coding region. For details about the procedure, see "Materials and Methods".

Each constructs contained the *ntp303* promoter, the firefly *luciferase*$^{+}$ (luc^{+}; Promega, Madison, WI) coding region, the *cauliflower mosaic virus* (*CaMV*) *35S* termination sequence, and different 5'-UTRs (Fig. 4A). Expression of the construct containing the *php303* 5'-UTR was compared with that of constructs containing control 5'-UTRs (syn44 5' and syn99 5') or the *ntp303* 5'-UTR. Syn44 5' and syn99 5' are two 5'-UTR sequences that provide efficient translation of transcripts during pollen development and pollen tube growth (Bate et al., 1996; Hulzink et al., 2002; chapter 2 this thesis). Each construct was introduced in mature petunia pollen by particle bombardment and the luciferase activity (representing the level of translation) was determined after 20 h of in vitro pollen tube growth. As is shown in Figure 4B, the luciferase activity level of the *php303* 5'-UTR construct was 40 or 7-fold higher than the activity level of the constructs containing the syn99 5' or syn44 5'-UTR, respectively. Compared with the luciferase activity level of the *ntp303* 5'-UTR construct, the luciferase activity level of the *php303* 5'-UTR construct was approximately 2-fold lower. From these data we conclude that the *php303* 5'-UTR exhibits the capacity to enhance translation of chimeric transcripts in petunia pollen tubes.

The 5'-UTR of *php303* reveals a high level of conservation with the *ntp303* and *lat51* 5'-UTRs

Because the *php303* and *ntp303* 5'-UTRs both enhances translation of chimeric transcripts in pollen tubes, we examined to what extent the 5'-UTR sequences were conserved. Sequence alignment analysis of the UTRs was performed using the software program AlignX from the Vector NTI package (Informax). No similarity was found between the 3'-UTRs (data not shown). However, alignment of the 5'-UTR sequences of *ntp303* and *php303* revealed a high level of similarity (Fig. 5). A high level of sequence similarity was also found between the *php303* and *lat51* 5'-UTRs. With regard to sequence similarity of all three 5'-UTRs, two highly conserved sequence regions could be distinguished. Furthermore, the *php303* and *ntp303* 5'-UTRs revealed the presence of two additional conserved sequence regions.

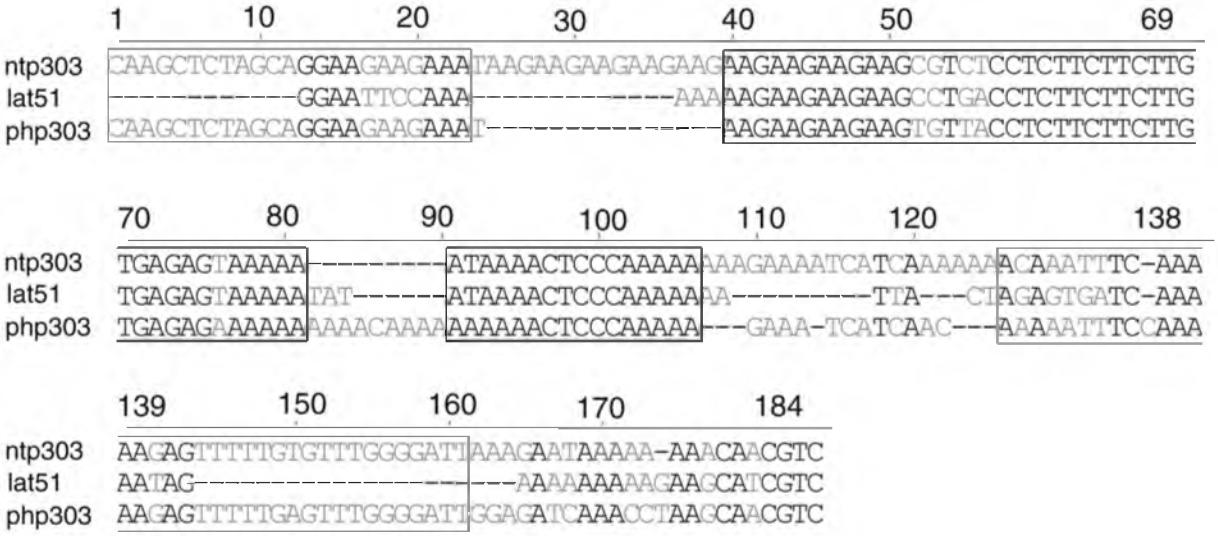

Figure 5. Alignment of the 5'-UTR nucleotide sequences of *php303*, *ntp303*, and *lat51*.

Nucleotides in bold indicate the consensus sequence. Highly conserved sequence regions are shown in the black-outlined boxes. The gray-outlined boxes comprise sequence regions that exhibit a high level of similarity between *php303* and *ntp303*. Dashes represent gaps introduced to improve the alignment. The positions of the nucleotides are indicated by numbers and are relative to the putative transcript start sites.

DISCUSSION

In an attempt to investigate to what extent genetic programs that determine delayed translation of stored pollen transcripts are conserved at the molecular level between related plant species, a comparative study has been performed for the tobacco pollen gene *ntp303* and homologous from petunia (*php303*) and tomato (*lat51*). Using a PCR-based strategy, we isolated a petunia homologue of the tobacco *ntp303* and tomato *lat51* genes. Based on the high level of amino acid sequence identity and the consequently positional conservation of sequences involved in post-translational modifications (glycosylation and myristoylation; Fig. 1A), we assume that *php303*, *ntp303* and *lat51* represent true homologous of each other.

The pattern of *php303* mRNA accumulation during pollen development and pollen tube growth (Fig. 2B) is very similar to that of *ntp303* transcripts. For *lat51*, no expression data is available. According to the differences in mRNA accumulation patterns during pollen development, pollen-expressed genes are divided in several classes (Stinson et al., 1987; Schrauwen et al., 1990). Because of the absence of *php303* transcripts in microspores and the

accumulation during pollen development, *php303* represents a late pollen gene. Accumulation of *php303* mRNA accumulation seems to be restricted to pollen (Fig. 2A). However, no detailed mRNA localization and reporter gene expression studies have been performed, yet. Therefore, we cannot rule out that *php303* is also expressed in non-pollen tissues. Previously, expression of the "pollen-specific" *ntp303* gene has been demonstrated to occur also in the anther and the pistil (chapter 3 this thesis).

Both the PHP303 and NTP303 proteins are abundantly present in germinating pollen, but their accumulation patterns are different. PHP303 is solely present in the cytosol and cell membrane (soluble) fraction (Fig. 3), whereas NTP303 is present in both the soluble and insoluble (cell wall) fractions (Wittink et al., 2000). However, the differences in localization might be artificial due to small variations of the extraction procedures. Because myristoylation may increase the affinity of a protein to the cell membrane (Cross, 1990; Chow et al., 1992), the presence of putative myristoylation sites in the PHP303 and NTP303 proteins (Fig. 1A) indicate that they both might be preferential associated to membranes. In addition, immunolocalization locates NTP303 predominantly at the cell membrane (Wittink et al., 2000).

The PHP303 protein starts to accumulate in detectable amounts during pollen development (Fig. 3), whereas NTP303 accumulates abundantly at the onset of pollen germination (Wittink et al., 2000). With regard to their respective mRNA accumulation patterns, both proteins exhibit a delay in their synthesis. Delayed translation of *php303* mRNA occurs during pollen development, whereas the onset of pollen germination determines the delayed translation of *ntp303* transcripts. Storage of mRNA during pollen development occurs for various pollen-expressed genes from different plant species (Mascarenhas, 1990, 1993; Schrauwen et al., 1990; Štorchová et al., 1994). Gene products that are synthesized from these stored transcripts are thought to be required for pollen tube growth.

In pollen tubes, the high level of expression of *php303* seems to be dependent on the 5'-UTR. The *php303* 5'-UTR led to a luciferase activity level that was many-fold higher than that of constructs containing control 5'-UTRs (Fig. 4B). With regard to structural properties of 5'-UTRs, a general accepted view is that lowering the potential energy of secondary structures (i.e. a more negative value of ΔG) within a 5'-UTR negatively influences translation efficiency by deterioration of scanning of the pre-initiation complex (for review, see Gallie, 1993; Fütterer and Hohn, 1996; Klaff et al., 1996). Despite the compulational prediction (RNAdraw software package; Matzura and Wennborg, 1996) that the secondary

structure of the *php303* 5'-UTR has a relative low potential energy (ΔG of –132 kJ mol^{-1}) which may results in a more negative effect on translation compared with the syn44 (ΔG of –51 kJ mol^{-1}) and syn99 (ΔG of –77 kJ mol^{-1}) 5'-UTRs, the *php303* 5'-UTR enabled efficient translation of chimeric transcripts during pollen tube growth. These data conclusively demonstrate that the *php303* 5'-UTR contains sequences that mediate efficient translation of pollen transcripts. The appearance of the PHP303 protein in late-bicellular pollen argues for enhancement activity of the *php303* 5'-UTR already during late phases of pollen development. In contrast, the 5'-UTR of the *ntp303* gene enhances translation of transcripts at the onset of pollen germination (Hulzink et al., 2002; chapter 2 and 3 this thesis). In petunia pollen tubes, the *ntp303* 5'-UTR enhanced translation of chimeric transcripts to a level that was more than two-fold higher than that of the *php303* 5'-UTR construct. This difference might be explained by the ΔG values of the 5'-UTR, i.e. the *php303* 5'-UTR has a more negative value of ΔG than the *ntp303* 5'-UTR, and therefore facilitate translation lesser efficient than the *ntp303* 5'-UTR does. Another interesting possibility for the observed differences in the activity of the 5'-UTR between *php303* and *ntp303* might be the variation in genetic programs that determine delayed translation of pollen transcripts in petunia and tobacco. We assume that the demand for a high level of protein during pollen tube growth can be accomplished by synthesis and storage of protein during late phases of pollen development and during pollen tube growth (in case of PHP303), or by accelerated protein synthesis at the onset of pollen germination (in case of NTP303). It might be that the differences in the time point of protein synthesis are reflected in the organization of the 5'-UTRs. Alignment of the 5'-UTR of *php303*, *ntp303* and *lat51* reveals a high level of sequence homology (Fig. 5), which includes regions that have been shown previously to direct a high level of *ntp303* mRNA translation in growing pollen tubes (Hulzink et al., 2002; chapter 2 this thesis). However, also differences exist. An example of a *ntp303* 5'-UTR sequence region that determines a high level of *ntp303* mRNA translation is a $(GAA)_8$ repeat. Within the *php303* and *lat51* 5'-UTRs, the AAG element is only repeated four times. It is assumable that such differences in the architecture of regulatory elements might lead to variation in the genetic programs that determine delayed translation of pollen mRNAs between closely related plant species.

During its development, the male gametophyte undergoes a complex program of cytological and cytochemical changes (for review, see Mascarenhas, 1989; Goldberg et al., 1993; McCormick, 1993). It is apparent that these development processes are conserved between different plant species during evolution. Consequently, it is obvious that the extent of

conservation of the developmental processes is reflected in the organization of the genetic programs that determine gene expression in pollen. At the transcriptional level, this becomes apparent in the conservation of several regulatory elements within the promoter of various pollen-expressed genes (Twell et al., 1991; Eyal et al., 1995; Tebbutt and Lonsdale, 1995; Weterings et al., 1995; Bate and Twell, 1998; Hamilton et al., 1998; Rogers et al., 2001). The presence of conserved putative regulatory sequences within the 5'-UTR of genes that originate from closely related plant species argues also for functional conservation of genetic programs that determine post-transcriptional regulation of pollen gene expression. In this respect, the existence of differences in gene expression patterns and the architecture and activity of 5'-UTRs between homologous pollen-expressed genes might provide valuable insight in the evolution of the genetic programs that determine post-transcriptional regulation of gene expression in pollen.

MATERIALS AND METHODS

Plant material

Greenhouse-grown plants of petunia (*Petunia hybrida* W138) were used as the source of genomic DNA, total RNA, and total protein. A transposon (dTph1)-inserted population of petunia (*Petunia hybrida* W115) plants was used for the identification and isolation of the *php303* gene. Pollen of different developmental phases was isolated from flower buds as previously described (Tupý et al., 1991; Van Herpen et al., 1992). Imbibed pollen was germinated in Read germination medium according to Read et al. (1993a, 1993b).

Isolation of the *php303* gene

A combination of a PCR-based transposon tagging and genome walking strategy on genomic DNA of respectively dTph 1-inserted and wild type petunia W138 plants led to the isolation of the *php303* gene. Initially, the transposon tagging strategy was used to obtain a functional knock out of a petunia homologue of *ntp303* (which is part of another study). Screening of a dTph1-inserted population of petunia W115 plants by PCR resulted in a 484-

bp-long DNA fragment. Primers that were used for the identification of the *ntp303* homologue DNA fragment were designed on the dTph1 transposon sequence (5'-CGGAATTCCACCAAGTAGCTCCGCCCCTG) and the 5'-part of the coding region of *ntp303* (27 nucleotides downstream of the *ntp303* translation initiation site; 5'-AGGGGTGATAGCTGAGGACCCTT). The DNA fragment was sequenced completely and used for the design of *php303*-specific primers. The subsequent isolation of the *php303* gene was performed using the commercial available Universal GenomeWalker kit (Clontech Laboratories, Palo Alto, CA). Briefly, genomic DNA from *Petunia hybrida* W118 was digested with five blunt cutting restriction enzymes, after which GenomeWalker adapters were ligated to the digested DNA fragments. PCR reactions were performed on the obtained genomic DNA libraries using a combination of adaptor and *php303* coding region-specific primers. The obtained PCR fragments were used for a second round of PCR, using a combination of nested adapter and gene-specific primers. The fragments were cloned into the pUC19 vector and sequenced completely. Finally, the *php303* gene sequence was annotated from the sequences of the different PCR fragments using the Vector NTI package (Informax).

Southern and Northern blot analysis

Genomic DNA and total RNA was isolated according Van Eldik et al. (1995). Twenty micrograms of genomic DNA was digested with EcoRI, BamHI and HindIII and fractioned on a 0.8 % agarose gel. Southern blotting was performed on Hybond-N^+ membranes according the manufacture's manual (Amersham, Buckinghamshire, UK)). Total RNA was blotted on Hybond-N^+ membranes using a protocol that has been described elsewhere (Hulzink et al., 2002). Hybridization of the Northern blots with a ^{32}P-labelled *php303* or ribosomal 18S probe and the washings of the blots have been described previously (Hulzink et al., 2002). Hybridization conditions of the Southern blots were similar as those of the Northern blots, with the exception of the hybridization temperature. Instead of 65°C the Southern blots were hybridized at 60°C. Low stringent washings were done for 30 min at 62°C using the following series of buffers: 2X SSC, 0.1% (w/v) SDS and 1X SSC, 0.1% (w/v) SDS. Moderate stringent washing required an additional step of 5X SSC, 0.1% (w/v) SDS. All blots were exposed to Kodak X-omat films (Eastman-Kodak, Rochester, NY) using two intensifying screens at –80°C.

Protein isolation and Western analysis

Isolation of soluble (cytosol and cell membrane) and insoluble (cell wall) total protein extracts from pollen and subsequent protein electrophoresis and Western blotting were performed according to Wittink et al. (2000). Bradford reagent (Bio-Rad Laboratories, Hercules, CA) was used for the determination of the concentration of total protein in the fractions (according the manufacture's instructions). Per sample, 50 micrograms of total protein was used for Western blotting. PHP303 protein detection was performed using a NTP303 C-terminal specific antibody (pAb/C; Wittink et al., 2000) at a 1:4,000 dilution along with a goat anti-rabbit AP conjugate (Pierce, Rockford, IL) at a 1:4,000 dilution. The blots were developed with 0.33 mg mL nitro blue tetrazolium (NBT) and 0.165 mg mL 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in alkaline phosphate buffer (100 mM Tris pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$).

5'-UTR reporter gene constructs and particle bombardment

Construction of the 5'-UTR reporter gene constructs containing the *ntp303* and control 5'-UTRs have been described elsewhere (Hulzink et al., 2002). These constructs contained the *ntp303* promoter (578-bp-long fragment), a modified version of the firefly *luciferase*$^+$ coding region (Promega), and the *CaMV 35S* termination sequence. To obtain a gene fusion construct with the *php303* 5'-UTR, the *ntp303* 5'-UTR was removed from the *ntp303* promoter, *luciferase*$^+$ coding region, and *CaMV 35S* 3'-UTR-containing construct using XhoI and NcoI restriction enzymes. The *php303* 5'-UTR was amplified by PCR using the *php303* genomic clone as template with the following primers (with restriction sites incorporated into the 5' end): 5'-GTGTCTCGAGCAAGCTCTAGCAGGAAGAAG (XhoI site underlined) and 5'-GTGTCCATGGGATCAAACCTAAGCAACGTC (NcoI site underlined). After PCR, the *php303* 5'-UTR was treated with XhoI and NcoI restriction enzymes and ligated in the construct lacking the *ntp303* 5'-UTR.

Particle bombardment and measurement of luciferase activity have been described elsewhere (Hulzink et al., 2002). Briefly, microcarriers were coated with test plasmid DNA (containing the *ntp303* promoter, the firefly *luciferase*$^+$ coding region, the *CaMV 35S* termination sequence, and the 5'-UTR of interest) and reference plasmid DNA (containing the *ntp303* promoter, the syn44 5'-UTR, the *R. reniformis luciferase* coding region (Promega),

and the *CaMV 35S* 3'-UTR), after which they were introduced into mature pollen by particle bombardment. After 20-h of pollen tube growth, the activity of the LUC^+ and *R*LUC luciferases was measured sequentially from a single sample using a luminometer containing two auto-injectors (Wallac 1420 VICTOR^2, PerkinElmer, Boston). Variability of the luciferase activity levels between independent experiments was normalized by calculation of the ratio of LUC^+:*R*LUC, resulting in a value that represents the relative luciferase activity per 10-s measuring time (RLA/10 s^{-1}). For each construct, at least six independent bombardments were performed.

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Chapter 5

In silico identification of putative regulatory sequence elements in the 5'-UTR of genes that are expressed during male gametogenesis

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ABSTRACT

Transcription of a large number of genes during pollen development results in the appearance of several populations of mRNAs that are distinctive with regard to their accumulation patterns. Similar mRNA populations are present in pollen of both mono- and dicotyledonous plant species, which argues for the conservation of genetic programs that determine pollen gene expression. The 5'-untranslated region (5'-UTR) of several of these mRNAs is of importance for directing pollen gene expression. The importance of 5'-UTR sequences for pollen gene expression and the apparent conservation of genetic programs in pollen, led to the hypothesis that the 5'-UTR of pollen-expressed gene from different plant species contains unique regulatory sequence elements. Computational analysis of pollen 5'-UTRs revealed the presence of a large number of elements that were significantly over-represented when compared to 5'-UTRs from sporophytic-expressed genes. Assembly of the pollen 5'-UTR elements led to the identification of various consensus elements. Several 5'-UTR elements appeared to be preferentially present in genes from dicots, wet-type stigma plants, or plants containing bicellular pollen. Functional implications of these observations are discussed.

INTRODUCTION

Gene expression comprehends a complex series of different regulatory steps. Gene expression studies in plants have mainly focused on mechanisms that underlie regulation of transcription. Consequently, the architecture and mode of action of promoter sequences from various genes in different plant systems have been investigated in much detail (for review, see Novina and Roy, 1996). Several of these studies revealed the existence of shared regulatory elements in the promoters of co-regulated genes coding for a variety of proteins. These elements are essential for directing expression of a set of genes under defined cellular conditions.

Despite the importance of transcription, it becomes increasingly evident that also post-transcriptional processes can exhibit a key-function in the regulation of plant gene expression

under defined cellular and environmental conditions (for review, see Fütterer and Hohn, 1996). Strictly seen, post-transcriptional processes comprehend all steps downstream of transcription, i.e. from pre-mRNA modification to protein turnover. However, the main determinant in post-transcriptional regulation comprises translation efficiency. In eukaryotes, control of translation efficiency often occurs at the translation initiation level by post-translational modification of translation initiation factors or by adaptation of individual mRNAs or populations of mRNAs (for review, see Pain, 1996; Kozak, 1999). In the latter instance, specific qualities of transcripts are important determinants for translation efficiency. In particular, the properties of the 5'-untranslated regions (5'-UTR) of mRNA molecules are important for directing translation efficiency. Examples are length (Gallie et al., 2000), secondary structure (Klaff et al., 1996; Gallie et al., 2000), upstream open reading frames (uORFs; Lukaszewicz et al., 1998; Wang and Wessler, 1998), and the composition of the sequence that surrounds the initiation codon (Geballe and Morris, 1994; Joshi et al., 1997). Moreover, an important regulatory quality of a 5'-UTR is the presence of regulatory elements that serve as interaction sites for antisense RNAs (Shayig, 1997; Hu et al., 1999) or RNA-binding proteins (for review, see Burd and Dreyfuss, 1994; Albà and Pagès, 1998).

In order to identify putative regulatory elements in the 5'-UTR of genes that are co-regulated under specific cellular and environmental conditions, we focused on genes that are expressed during the development and germination of the male gametophyte or pollen. During pollen development, a large number of genes is transcribed (Willing and Mascarenhas, 1984; Willing et al., 1988) which results in the presence of several populations of mRNAs that differ in their accumulation pattern (Stinson et al., 1987; Schrauwen et al., 1990). These mRNAs are used for de novo translation or are stored for their utilization during following phases of pollen maturation or pollen tube growth (Čapková et al., 1988; Schrauwen et al., 1990). For several of these mRNAs, 5'-UTR sequences have been shown to alter gene expression at the transcriptional (Curie and McCormick, 1997) or post-transcriptional level (Bate et al., 1996; Hulzink et al., 2002; chapter 2 and 3 this thesis). The presence of identical mRNA accumulation patterns in pollen from different plant species clearly indicates conservation of different genetic programs that underlie pollen gene expression. Because of the apparent conservation of genetic programs between different plant species and the important role of the 5'-UTR for pollen gene expression, we hypothesize that the 5'-UTR of pollen-expressed genes shares unique regulatory elements.

In order to identify putative regulatory elements that are preferentially present (over-represented) in the 5'-UTR of pollen-expressed genes, a computational analysis has been

carried out. Therefore, two different sequence datasets were created containing 5'-UTR sequences of pollen-expressed genes (pollen sequences) and genes that have been isolated from sporophytic tissues (reference sequences). These sequence sets were used for a statistical study for the over-representation of sequence elements (oligo analysis; Van Helden et al., 1998, 2000a). The pollen-expressed genes that were used for the oligo analysis were derived from various plant species. Although genetic programs in pollen are conserved between different plant species, it may be assumed that defined putative regulatory elements are preferentially preserved in plant species that exhibit a certain degree of similarity on basis of their taxonomic classification or the presence of morphological features. Therefore, we examined also the correlation between the presence of defined elements and their origin from plant species that are distinctive on basis of either the number of cotyledons (mono- or dicots), stigma-type (wet or dry), or pollen-type (bicellular or tricellular).

RESULTS

The 5'-UTR of pollen-expressed genes contains a large number of over-represented sequence elements

In order to investigate whether the 5'-UTR of pollen-expressed genes contains over-represented sequence elements (oligonucleotides), two datasets containing 5'-UTR sequences of pollen-expressed genes (Table I; pollen sequences) and genes that have been isolated from sporophytic tissues (reference sequences) were made. All oligonucleotide occurrences were counted in the pollen dataset and their statistical significance was estimated by using the oligonucleotide data from the reference sequences (for a detailed description of the oligo analysis, see Van Helden et al., 1998, 2000b).

Table II shows the hexanucleotides that were significantly over-represented in the pollen sequences compared to the reference sequences. The first four columns represent the results of the total number of pollen sequence elements that were over-represented. Among the 4096 possible hexanucleotides, the oligo analysis selected 31 sequence elements as preferentially present in the 5'-UTR of pollen-expressed genes (Table II). Similar results were obtained for penta-, hepta-, and octanucleotides (for these data, see the web site

Species	Clone	Ac. Nr.	Species	Clone	Ac. Nr.
A.t.		AJ002280	N.t.	nsk59	AJ002315
A.t.	atbnh (T5A14.2)	AJ249211	N.t.	nsk91	AJ224163
A.t.	atprf4	U43594	N.t.	nsk111	AJ002314
A.t.	at59 (a2)	U83619	N.t.	ntf4	X83880
A.t.	gmd1	AF195140	N.t.	nthsp18p	X70688
A.t.	lpd2	AF228638	N.t.	ntplim1a	AF197567
A.t.	pab5	M97657	N.t.	ntplim1b	AF197568
A.t.	pfn4	U43324	N.t.	ntpro2	X93465
A.t.	prf1	U43590	N.t.	ntpro3	X93466
A.t.	prf2	U43591	N.t.	ntp303	X69440
A.t.	profilin1	U43325	N.t.	ntsut3	AF149981
A.t.	profilin2	U43326	N.t.	nt59	U85646
A.t.	rab2	Y09314	N.t.	plim2	AF116851
A.t.	rac2	AF107663	N.t.	pronp1	AJ130969
A.t.	rkf1	AF024648	N.t.	p18	AJ004957
A.t.	rop1at	U49971	N.t.	rop1	AJ222545
A.t.	rop4	AF031428	N.t.	tac25	X63603
A.t.	rop6	AF031427	N.t.	tobaldh2a	Y09876
A.t.	suc1	X75365	N.t.	tobpdc2	X81855
B.n.	bp4a	X52874	N.t.	tp5	AJ250431
B.n.	bp4c	X52874	N.t.	tp10 (g10)	X67159
B.n.	bp19	X56195	N.t.	136.1	U20490
B.n.	hmga	AF127919	O.s.	ps1	Z16402
B.n.	rbp1	AF094825	O.s.	udpgase	AF249880
B.n.	sta39-3	L47351	P.h.	pgps/d1	AF049917
B.n.	sta39-4	L47352	P.h.	pgps/d2	AF049918
B.n.	sta44-4	L19879	P.h.	pgps/d3	AF049919
B.r.	bcp1	X68209	P.h.	pgps/d4	AF049920
B.r.	bgp1	X68210	P.h.	pgps/d6	AF049922
B.r.	brar1	AB032260	P.h.	pgps/d8	AF049924
B.r.	brar2	D63154	P.h.	pgps/d10	AF049926
H.a.	plim1a (sf3)	AF187104	P.h.	pgps/d11	AF049927
H.a.	plim2	AF047353	P.h.	pgps/d12	AF049928
H.a.	sf16	X74772	P.h.	pgps/d14	AF049930
H.a.	sf17	X81997	P.h.	pgps/nh21	AF049937
H.b.	trx	AF159385	P.h.	pmt1	AF061106
I.t.	isp11	U29432	P.h.	php303 (p303)	AF479568
L.l.	a23	AF077629	P.i.	ppc1 (pcpc22)	L27101
L.l.	ltp	AF171094	P.i.	prk1	L2731
L.l.	p35	AB012694	P.p.	-	AB013353
L.l.	y5-7	AF088901	P.s.	rop1ps	L19093
L.l.	14-3-3	AF191746	S.b.	sbpk	X97980
L.l.	-	L18909	S.b.	-	SB401
L.l.	-	L18911	S.c.	-	AF161330
L.l.	-	Z17328	S.t.	invge	AJ133765
L.p.	trx	AF159387	S.t.	invgf	AJ133765
L.c.	lat51	AF394216	T.p.	tpc70	U04298
L.e.	lat52	X15855	Z.m.	hstb	X82943
L.e.	lat56	X56487	Z.m.	hsp70	X03658
L.e.	lat59	X56488	Z.m.	mpex 2	AF159297
L.e.	leprk1	U58474	Z.m.	mpex1	Z34465
L.e.	leprk2	U58473	Z.m.	pg1	X57627
L.e.	leprot1	AF014808	Z.m.	tua1 (mg19/6)	X15704
L.e.	leprot2	AF014809	Z.m.	tua4	X63179
L.e.	lpex (pex1)	AF159296	Z.m.	tua6	X63179
M.s.	po2	U28149	Z.m.	tub3	X74654
M.s.	po22	U40387	Z.m.	tub4	X74655
M.s.	p65	U28148	Z.m.	tub5	X74656
M.s.	p73	U20431	Z.m.	zmabp1	
N.a.	nacsld1	AF304375	Z.m.	zmc5	Y13285
N.a.	nagsl1	AF304372	Z.m.	zmmads1	AF112148
N.s.	nsaap1	U31932	Z.m.	zmmads2	AF112149
N.t.	eif4a8	X79004	Z.m.	zmpro1	X73279
N.t.	jd1	AF316320	Z.m.	zmpro2	X73280
N.t.	npg1 (tp27)	X71017 / X71020	Z.m.	zmpro3	X73281
N.t.	nsk6	Y08607	Z.m.	zm13	S44171

Table I. List with pollen-expressed genes that were used for the oligo analysis.

The first column shows the plant species: A.t.; *Arabidopsis thaliana*, B.c.; *Brassica campestris*, B.n.; *Brassica napus*, B.r.; *Brassica rapa*, H.a.; *Helianthus annuus*, H.b.; *Hordeum bulbosum*, I.t.; *Ipomoea trifida*, L.l.; *Lilium longiflorum*; L.p.; *Lolium perenne*, L.e.; *Lycopersicon esculentum*, M.s.; *Medicago sativa*, N.a.; *Nicotiana alata*, N.s.; *Nicotiana sylvestris*, N.t.; *Nicotiana tabacum*, O.s.; *Oryza sativa*, P.h.; *Petunia hybrida*, P.i.; *Petunia inflata*, P.p.; *Pyrus pyrifolia*, P.s.; *Pisum sativa*, S.b.; *Solanum berthaultii*, S.c.; *Solanum chacoense*, S.t.; *Solanum tuberosum*, T.p.; *Tradescantia paludosa*, and Z.m.; *Zea mays*. The second column represents the gene / clone names. The third column shows the GenBank accession numbers.

http://rsat.ulb.ac.be/rsat/). The majority of the over-represented oligonucleotides were A-rich, i.e. more than 80% of the oligonucleotides contained four or more A-residues. The most significant oligonucleotide was AAAAAA, which was found in 74 occurrences (O_{occ}) when randomly 20.4 would be expected (E_{occ}). Taking the total number of possible oligonucleotides

	Oligo	O_{occ}	E_{occ}	P_{occ}	Sig_{occ}	O_{ms}	E_{ms}	P_{ms}	Sig_{ms}
1	aaaaaa	74	20.4	$2.8e^{-20}$	15.9	41	19.4	$1.8e^{-06}$	2.1
2	aaataa	38	8.3	$4.4e^{-14}$	9.8	28	8.1	$8.7e^{-09}$	4.4
3	aaaaat	41	11.5	$1.1e^{-11}$	7.3	29	11.2	$1.7e^{-06}$	2.1
4	aaaata	30	8.0	$2.3e^{-09}$	5.0	23	7.9	$3.9e^{-06}$	1.8
5	aataaa	31	8.5	$2.4e^{-09}$	5.0	22	8.4	$3.1e^{-05}$	0.9
6	gaaaaa	49	19.1	$7.6e^{-09}$	4.5	36	18.1	$3.5e^{-05}$	0.8
7	caaaaa	42	15.4	$1.7e^{-08}$	4.2	30	14.8	$1.3e^{-04}$	0.3
8	aaagga	22	5.1	$2.3e^{-08}$	4.0	22	5.0	$6.7e^{-09}$	4.6
9	aaggaa	23	5.8	$5.9e^{-08}$	3.6	20	5.7	$1.3e^{-06}$	2.3
10	ataaaa	27	8.3	$2.1e^{-07}$	3.1	20	8.1	$1.8e^{-04}$	0.1
11	taaaaa	28	9.0	$3.3e^{-07}$	2.9	21	8.8	$2.0e^{-04}$	0.1
12	aaaaag	40	16.2	$4.3e^{-07}$	2.8	30	15.5	$2.9e^{-04}$	-0.1
13	ggaaaa	22	6.1	$4.7e^{-07}$	2.7	19	6.0	$9.1e^{-06}$	1.4
14	caaata	15	3.3	$2.4e^{-06}$	2.0	13	3.3	$3.2e^{-05}$	0.9
15	aataag	11	1.9	$4.0e^{-06}$	1.8	10	1.8	$1.9e^{-05}$	1.1
16	aaaaac	34	14.2	$6.0e^{-06}$	1.6	24	13.7	$4.7e^{-03}$	-1.3
17	atcaaa	23	7.8	$7.9e^{-06}$	1.5	18	7.7	$6.6e^{-04}$	-0.4
18	aagaca	17	4.8	$1.3e^{-05}$	1.3	14	4.8	$3.3e^{-04}$	-0.1
19	aaaaga	37	17.2	$2.2e^{-05}$	1.0	30	16.4	$7.3e^{-04}$	-0.5
20	ctttga	15	4.1	$2.5e^{-05}$	1.0	13	4.1	$2.3e^{-04}$	0.0
21	aagaag	37	17.7	$3.9e^{-05}$	0.8	21	16.8	$1.7e^{-01}$	-2.8
22	taaagg	9	1.6	$4.5e^{-05}$	0.7	9	1.6	$3.7e^{-05}$	0.8
23	caataa	15	4.3	$4.9e^{-05}$	0.7	14	4.3	$1.1e^{-04}$	0.3
24	ccaaaa	25	10.1	$5.0e^{-05}$	0.7	22	9.8	$2.9e^{-04}$	-0.1
25	ttttaa	20	7.3	$8.1e^{-05}$	0.5	16	7.2	$2.3e^{-03}$	-1.0
26	aaggag	16	5.1	$8.4e^{-05}$	0.5	14	5.0	$5.4e^{-04}$	-0.3
27	tatcaa	13	3.6	$9.8e^{-05}$	0.4	11	3.6	$9.7e^{-04}$	-0.6
28	ttggaa	9	1.9	$1.3e^{-04}$	0.3	9	1.8	$1.1e^{-04}$	0.3
29	ggaatt	13	3.8	$1.9e^{-04}$	0.1	12	3.8	$4.7e^{-04}$	-0.3
30	aggaaa	20	7.8	$1.9e^{-04}$	0.1	18	7.7	$6.6e^{-04}$	-0.4
31	aaacaa	31	15.0	$1.9e^{-04}$	0.1	22	14.4	$2.8e^{-02}$	-2.1

Table II. Over-represented sequence elements in the 5'-UTR of pollen-expressed genes.

First column represents the over-represented pollen 5'-UTR sequence elements (oligonucleotides). O_{occ}; Observed occurrences, E_{occ}; Expected occurrences, P_{occ}; Probability occurrences, Sig_{occ}; Significance index occurrences, O_{ms}; Observed matching sequences, E_{ms}; Expected matching sequences; P_{ms}; Probability matching sequences, and Sig_{ms}; Significance index matching sequences. All sequence elements with $Sig_{occ} > 0$ were selected. For description of the parameters, see "Materials and Methods".

in each dataset into account (4096), we conversed the occurrence probability value to the occurrence significance index (sig_{occ}). For the oligonucleotide AAAAAA, the sig_{occ} was 15.9 meaning that a hexanucleotide with a similar high significance value is expected to occur in $10^{15.9}$ datasets of random sequences. High significant values were also found for several AAAAAA single-substitution variants. To examine whether the over-represented oligonucleotides were present in a low or high copy number within the pollen sequences, the matching sequence significance (sig_{ms}) was determined (Table II). The highest sig_{ms} was observed for AAAGGA. This element had the lowest copy number possible, i.e. it was present as a single signal within all the selected pollen sequences. To examine whether the over-represented oligonucleotides exhibited a positional bias within the pollen sequences, the distribution of each hexanucleotide was determined statistically (Table III). With regard to the threshold value of 64.64, none of the oligonucleotides exhibited a preferential localization in any of the pollen 5'-UTRs

	Oligo	X_2		Oligo	X_2		Oligo	X_2
1	aaaaaa	45.69	12	aaaaag	27.75	23	caataa	10.76
2	aaataa	14.74	13	ggaaaa	15.36	24	ccaaaa	39.24
3	aaaaat	12.60	14	caaata	22.28	25	ttttaa	19.42
4	aaaata	21.60	15	aataag	18.03	26	aaggag	16.95
5	aataaa	11.56	16	aaaaac	26.90	27	tatcaa	36.75
6	gaaaaa	21.50	17	atcaaa	15.91	28	ttggaa	15.75
7	caaaaa	27.37	18	aagaca	9.84	29	ggaatt	12.22
8	aaagga	15.08	19	aaaaga	12.28	30	aggaaa	18.49
9	aaggaa	32.21	20	ctttga	11.19	31	aaacaa	37.20
10	ataaaa	10.05	21	aagaag	24.65			
11	taaaaa	23.14	22	taaagg	19.42			

Table III. Distribution analysis of the pollen 5'-UTR sequence elements.

Analysis of the distribution of each over-represented sequence elements was performed with the program "position analysis" (Van Helden et al., 200a; for a description of the methodology, see "Materials and Methods"). Chi-square values (X_2) were calculated using the parameters df = 30; α = 0.000244. Sequence elements with $X_2 \leq 64.64$ did not exhibited a significant biased position within the pollen sequences.

In summary, these data clearly demonstrate that several sequence elements are preferentially present in the 5'-UTR of pollen-expressed genes from different plant species. Within the pollen 5'-UTR, the over-represented sequence elements are randomly distributed and are inclined to be present in a low copy number.

Assembly of oligonucleotides gives rise to several consensus elements

Several of the identified oligonucleotides are related by sequence similarity and their mutual overlap might reveal consensus elements. In order to identify these consensus elements, sequence-related elements were assembled using the program "pattern assembly" (Van Helden et al., 1998). Table IV shows the presence of several pollen 5'-UTR consensus elements after assembly of sequence-related over-represented oligonucleotides. With regard to their significance index, several consensus sequences are highly over-represented in the 5'-UTR of pollen-expressed genes.

	Oligo	Sig_{occ}		Oligo	Sig_{occ}		Oligo	Sig_{occ}
4	aaaata.....	5.0	1	aaaaaa	15.9	22	taaagg	0.7
7	caaaaa.....	4.2	3	aaaaat	7.3	8	.aaagga	4.0
14	caaata.....	2.0	4	aaaata	5.0	19	.aaaaga	1.0
1	.aaaaaa....	15.9	6	gaaaaa	4.5	9	..aaggaa	3.6
2	.aaataa....	9.8	7	caaaaa	4.2	26	..aaggag	0.5
23	.caataa....	0.7	10	ataaaa	3.1	30	...aggaaa	0.1
31	.aaacaa....	0.1	11	taaaaa	2.9	6	....gaaaaa	4.5
5	..aataaa...	5.0	12	aaaaag	2.8	13	....ggaaaa	2.7
15	..aataag...	1.8	16	aaaaac	1.6	**C**	**taaaggaaaa**	**4.5**
10	..ataaaa...	3.1	19	aaaaga	1.0			
17	...atcaaa..	1.5	**C**	**aaaaaa**	**15.9**			
6	....gaaaaa.	4.5						
11	....taaaaa.	2.9						
3	.....aaaaat	7.3						
12	.....aaaaag	2.8						
16	.....aaaaac	1.6						
C	**caaataaaaat**	**15.9**						
7	caaaaa	4.2	27	tatcaa.	0.4	12	aaaaag	2.8
24	ccaaaa	0.7	10	.ataaaa	3.1	15	aataag	1.8
C	**ccaaaa**	**4.2**	17	.atcaaa	1.5	21	aagaag	0.8
			C	**tatcaaa**	**3.1**	26	aaggag	0.5
						C	**aagaag**	**2.8**

Table IV. Pattern assembly of the pollen 5'-UTR sequence elements.

First column of each series shows the gene numbers as given in Table I. The "oligo" columns represent the pollen 5'-UTR sequence elements with their respective significant indexes (column 3; Sig_{occ}). C; consensus sequence element.

Preferential present of 5'-UTR elements in pollen genes from dicots, plants containing a wet-type stigma, or bicellular pollen

The pollen-expressed genes that were used for the 5'-UTR analysis originated from 25 different plant species (Table I). On basis of their taxonomic classification, these plants could be classified in mono- and dicotyledonous species. Moreover, the plant species could be grouped on basis of their stigma type (wet or dry) and pollen type (bi- or tricellular).

	Oligo	X_2 Cotyl	X_2 Stigma	X_2 Pollen		Oligo	X_2 Cotyl	X_2 Stigma	X_2 Pollen
1	aaaaaa	**9.00**	1.23	0.11	17	atcaaa	2.13	**9.26**	**11.14**
2	aaataa	3.83	0.29	0.00	18	aagaca	0.94	2.86	**5.15**
3	aaaaat	**13.90**	2.63	0.32	19	aaaaga	**10.71**	**4.12**	3.27
4	aaaata	**6.63**	0.08	1.26	20	ctttga	0.03	**3.93**	2.00
5	aataaa	**9.77**	0.80	0.00	21	aagaag	**6.70**	0.00	1.36
6	gaaaaa	**10.68**	**7.24**	3.79	22	taaagg	0.13	1.72	0.44
7	caaaaa	**10.31**	1.97	**4.34**	23	caataa	**5.58**	0.33	1.57
8	aaagga	**4.18**	3.26	**5.22**	24	ccaaaa	2.44	**5.08**	**7.54**
9	aaggaa	1.79	**5.45**	3.60	25	ttttaa	**6.47**	1.54	0.27
10	ataaaa	**8.79**	0.86	0.00	26	aaggag	2.65	0.32	1.84
11	taaaaa	**6.70**	0.80	0.20	27	tatcaa	2.28	0.67	2.06
12	aaaaag	**5.34**	**4.49**	3.76	28	ttggaa	3.17	1.07	0.26
13	ggaaaa	1.51	2.64	1.75	29	ggaatt	0.03	0.10	0.10
14	caaata	0.23	0.33	1.57	30	aggaaa	1.18	3.78	2.78
15	aataag	3.48	0.06	0.05	31	aaacaa	2.25	0.08	0.00
16	aaaaac	**4.19**	**5.12**	3.34					

Table V. Correlation between over-represented sequence elements and their presence in the 5'-UTR of pollen-expressed genes that originates from mono- or dicots, plants with wet- or dry-type stigmas, or plants bearing bi- or tricellular pollen.

Statistic analysis of the extent of preferential presence of over-represented sequence elements within three different biological categories: number of cotyledons (cotyl), stigma-type (stigma), and pollen-type (pollen). For a description of the methodology, see "Materials and Methods". Chi-square values (X_2) were determined using the parameters df = 1; $\alpha \leq 0.05$. Sequence elements with $X_2 \geq 3.84$ (bold) exhibited a preferential presence in genes from dicots, plants with wet-type stigmas, or plants containing bicellular pollen.

We were interested whether the 5'-UTR elements were preferentially conserved in pollen genes that were derived from species containing one or two cotyledons, a wet or dry-type stigma, or bi- or tricellular pollen. Using chi-square statistics, a strong ($\alpha \leq 0.05$) correlation

existed between 15 of the 31 different over-represented oligonucleotides and their presence in pollen sequences from dicotyledonous plants species (Table V). With regard to the stigma-type, the analysis revealed the preferential presence of eight oligonucleotides in pollen genes from wet-type stigma plants. Wet-type stigmas are covered with a liquid secretion layer, whereas dry stigmas are covered with less or no secretion material (Heslop-Harrison and Shivanna, 1977). The biological criterion "pollen type" revealed a strong correlation between five oligonucleotides and their presence in pollen 5'-UTRs from bicellular pollen plants. Sperm cells in tricellular pollen are formed during pollen maturation, whereas sperm cells in bicellular pollen are arranged during pollen tube growth (Brewbaker, 1967). As shown in Table V, several oligonucleotides were not preserved in specific plant species. In summary, these results clearly demonstrate that several but not all pollen 5'-UTR elements are preferentially present in genes from dicotyledonous, wet-type stigma, or bicellular pollen-type plant species.

DISCUSSION

A systematic approach based on the statistical analysis of oligonucleotide occurrences has led to the identification of a large number of elements that are significantly over-represented in the 5'-UTR of pollen-expressed genes compared to a reference set of 5'-UTRs. It is obvious that the choice of appropriate reference and test (pollen; Table I) datasets is an important determinant for the outcome of the analysis. Genes from both datasets were selected on basis of the origin of their respective cDNAs (male gametophytic or sporophytic tissues), without a priori consideration of the composition of the 5'-UTRs. The extent of expression of pollen genes during pollen development and tube growth was ascertained by their description in literature. Although we have no detailed expression information for the reference genes, the presented oligo analysis was able to determine 31 different elements that were significantly preserved in the pollen sequences (Table II). Within the pollen 5'-UTRs, the over-represented elements were randomly distributed (Table IV) and were preferentially present in a low copy number (Table II).

Successful identification of over-represented elements has been fulfilled for promoter (Van Helden et al., 1998, 2000b) and 3'-UTR (Jacobs-Anderson and Parker; 2000, Van

Helden et al., 2000a) sequences of co-regulated yeast genes. To our knowledge, the present study is the first report describing the in silico identification of conserved 5'-UTR elements in co-regulated plant genes. Although many sequence elements are significantly correlated to the 5'-UTR of pollen-expressed genes, their statistical significance does not necessarily implicate a functional meaning. However, several observations indicate that some of the pollen elements might exhibit pollen-related regulatory properties. Figure 1 shows a schematic

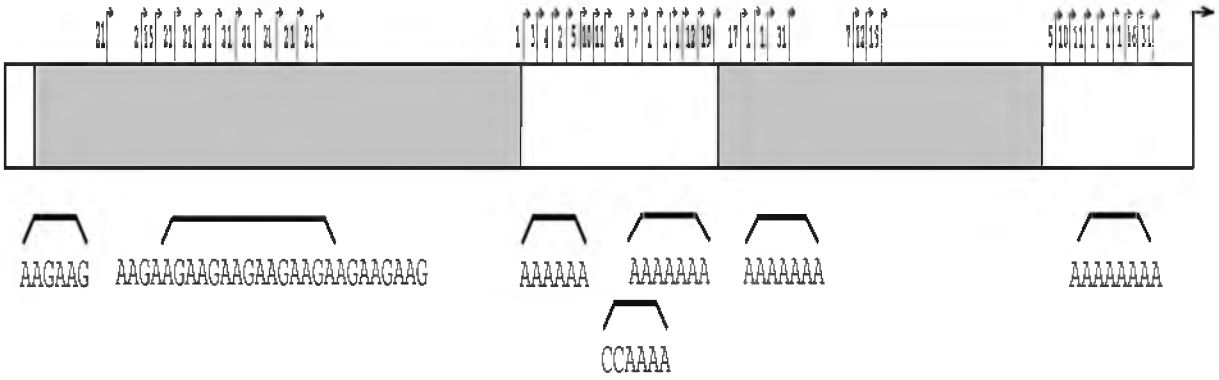

Figure 1. Schematic representation of the distribution of over-represented sequence elements in the 5'-UTR of the pollen-expressed gene *ntp303*.

Distribution of over-represented sequence elements is presented above the graphic representation of the *ntp303* 5'-UTR. The small arrows indicate the start of a sequence element. The numbers correspond to the individual sequence elements as presented in Table II. Sequences below the *ntp303* 5'-UTR indicate the position of several consensus sequence elements as presented in Table IV. The gray regions represent the 5' (left) and 3' (right) regulatory regions of the *ntp303* 5'-UTR (see text for description). The arrow at the 3'-end of the *ntp303* 5'-UTR indicates the position of the translation initiation site.

representation of the 5'-UTR of the pollen-expressed gene *ntp303*. A sequence region at the 5'-end of the 5'-UTR has previously been shown to determine translation efficiency, whereas a sequence region at the 3'-end was found to be important for mRNA stability (Hulzink et al., 2002; chapter 2 this thesis). Mutual overlap of several of the pollen elements in the *ntp303* 5'-UTR revealed the presence of extended sequence regions (Fig. 1). Because some of these regions reside within the functional *ntp303* 5'-UTR regions, we assume that several of the assembled pollen sequence regions comprise regulatory elements. Moreover, pattern assembly analysis of the pollen elements demonstrated the presence of several consensus elements in the 5'-UTR of pollen-expressed genes (Table III). As is shown in Figure 1, some of these patterns are also localized in the functional regions of the *ntp303* 5'-UTR. Since regulatory sequence elements are often concentrated as short and highly conserved core

elements (for review, see Novina and Roy, 1996), we assume that the consensus elements in the functional *ntp303* 5'-UTR regions resemble regulatory sequences. A representative example of such a regulatory element is the consensus sequence AAGAAG. This sequence is a repeat element within the 5'-functional region of the *ntp303* 5'-UTR. Deletion analysis has revealed the importance of this repeat element for directing pollen gene expression (Hulzink et al., 2002; chapter 2 this thesis). In this respect, the identification of the AAGAAG sequence as a pollen 5'-UTR-conserved element provides additional clues for its regulatory function. In summary, the present data clearly demonstrate the existence of pollen gene 5'-UTR sequence elements. For several of these pollen elements, we have clues that they represent regulatory sequence elements. However, it is apparent that a detailed functional study of the over-represented 5'-UTR sequence elements is necessary to obtain more insight in their putative regulatory role for pollen gene expression.

The pollen-expressed genes that were used in the present study were obtained from various plant species that can be grouped on basis of their taxonomic classification into mono- and dicots (Table I). Remarkably, many pollen 5'-UTR elements revealed a preferential presence for pollen genes that were derived from dicotyledonous plant species, whereas none of the elements exhibited a significant over-representation in monocots (Table V). These results indicate co-evolution of putative regulatory elements in the 5'-UTR of pollen-expressed genes and of genetic programs that determine pollen gene expression in dicots. It is plausible that the preferential preservation of 5'-UTR elements is reflected by specific properties of dicots that are absent in monocots. In addition, preferential presence of pollen 5'-UTR elements was also observed for pollen-expressed genes from plant species containing wet-type stigmas or bicellular pollen. Such a clear relationship indicates a regulatory role of such 5'-UTR elements for pollen gene expression in species with wet-type stigmas or bicellular pollen. Interestingly, the presence of many other pollen 5'-UTR elements was not related to the number of cotyledons, stigma-type, and pollen-type. These elements might play a role in the regulation of pollen gene expression independently of the genetic background of the plant species.

It is obvious that in silico identification of conserved sequence elements in the 5'-UTR of co-regulated genes has to be validated experimentally. Nevertheless, computational identification of preserved 5'-UTR elements provides useful indications for new functional studies. Although gene expression studies in pollen have been prosperous in several ways, a systematic analysis of regulatory regions of the growing number of isolated pollen-expressed

genes was still lacking. In this respect, the present study has provided new intriguing functional and evolutionary clues with regard to the regulation of pollen gene expression.

MATERIALS AND METHODS

5'-UTR sequence databases

The 5'-UTR sequences of pollen-expressed genes (pollen sequences) were manually collected from the GenBank database. The pollen 5'-UTR dataset consists of 5'-UTR sequences from 132 different genes that are highly expressed in mature pollen or pollen tubes. Expression of the genes in pollen or pollen tubes was determined by means of their literature or GenBank entry description. Genes related to pollen coat proteins or pollen allergens have been excluded from the pollen sequences. The total number of nucleotides in the pollen 5'-UTR dataset is 16,645. The average length of the pollen 5'-UTRs is 126 nucleotides; the smallest UTR sequence consists of six nucleotides, whereas the longest UTR is 620 nucleotides in length.

The detection of over-represented oligonucleotides within the pollen sequences relies on a prior definition of the background model, which will serve to estimates the random expectation for each oligonucleotide. The background model that we have used for the analysis was a non-pollen 5'-UTR database (reference sequences). Reference sequences were obtained from the GenBank database (March 2001) by selecting the first 1076 cDNA entries that did not contain the key words 'pollen', 'gametophyte', 'flower', and 'bud'. Extraction of the 5'-UTRs from the cDNA sequences was done manually. With regard to the extraction of 5'-UTR sequences, the complete sequence upstream of the translation initiation site was selected. This procedure resulted in the collection of 113,481 nucleotides. The average length of the reference 5'-UTRs is 105 nucleotides; the smallest UTR sequence is 14 nucleotides in length, whereas the longest UTR consists of 1396 nucleotides.

Oligo analysis

A pattern discovery approach (oligo analysis; Van Helden et al., 1998, 2000a) was used to detect significant over-represented oligonucleotides within the pollen sequences on basis of statistical analysis of oligonucleotide occurrences (for detailed description, see Van Helden et al., 1998). Oligo analysis was first applied to the reference sequence set in order to calculate the expected frequency of each oligonucleotide. The expected frequencies were used to determine the number of expected occurrences for each oligonucleotide in the set of pollen sequences. The binomial occurrence probability (P_{occ}) was calculated to compare the observed (O_{occ}) and expected oligonucleotide (E_{occ}) occurrences within the pollen sequences; i.e. P_{occ} represents the probability to have n (= number of observed occurrences) or more occurrences given the expected number of occurrences. All oligonucleotides were selected that exhibited a positive value for their significance index ($Sig_{occ} > 0$).

The number and expected number of pollen sequences that contained at least one occurrence of an oligonucleotide were also determined. Therefore, the observed matching sequences (O_{ms}) and expected matching sequences (E_{ms}) were calculated (for procedure, see the web site http://rsat.ulb.ac.be/rsat/). Subsequently, the matching sequence probability (P_{ms}) was calculated to compare the observed and expected matching pollen sequences. P_{ms} represents the probability to have m (= number of observed matching sequences) or more matching sequences with at least one oligonucleotide occurrence given the expected matching sequences. The matching sequence significance index (Sig_{ms}) was identified to take the number of possible oligonucleotides into account. $Sig_{ms} > 0$ means that the respective oligonucleotide is not solely present as a single copy pattern within the pollen sequences.

Using the 1076 non-pollen gene sequences as reference, a problem of statistical sampling was observed. Some oligonucleotides were only present in the pollen sequences. Consequently, their probability was estimated to be zero and their respective significance was infinite, even if they were found in a low copy number in the pollen sequences. The problem occurred because the reference sequence set was too small to reflect all possibilities. It is likely that these oligonucleotides will appear in much larger reference data sets. In order to circumvent this problem with the current reference sequence set, pseudo-weights were used: i.e. the oligonucleotide frequencies calculated from the reference sequence set contributed for 90% to the estimation of prior oligonucleotide probabilities, and the remaining 10% were left for the potential presence of additional oligonucleotides in the pollen sequences.

Analysis of oligonucleotide distributions within the pollen sequences was performed with the program 'position analysis' (Van Helden et al., 2000a). Occurrences were regrouped by intervals of 20 base pairs. Given the sequence sizes, the positional distributions contained 31 classes with a class interval of 20. The expected distribution was calculated according to a homogeneous model (flat distribution for each pattern). Observed and expected positional distributions were compared using the chi-squared statistics. To take the number of patterns (N=4096) into account, the threshold was adapted according to the Bonferoni rule which recommends a first error risk $\alpha < 1/N$. The degrees of freedom (df) of the chi-square depend on the number of position classes (c), which in turn depends on the class interval. The resulting threshold ($\alpha = 0.000244$) was 64.64 for the class interval of 20 base pairs.

To examine to what extent the presence of defined oligonucleotides was correlated to the number of cotyledons, stigma-type, or pollen-type, chi-square statistics were applied (df=1; $\alpha \leq 0.05$ and ≥ 0.01).

Availability

The Regulatory Sequence Analysis Tools are available via their web interface at the URL http://rsat.ulb.ac.be/rsat/. The complete sets of data and calculation procedures are available on the same site.

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Chapter 6

Summary and concluding remarks

The evolution of angiosperms or flowering plants during the last 100 million years has led to the appearance of more than 250,000 different species nowadays. Although the remarkable variety between flowering plants is clearly reflected in the diversity of pollination and mating systems, several reproductive processes are conserved. For example, the development of the male gametophyte or pollen is characterized by cytological changes that are highly similar between mono- and dicotyledonous plant species (for review, see Mascarenhas, 1989; Scott et al., 1991; Bedinger, 1992; Goldberg et al., 1993; McCormick, 1993). One of the most noticeable cellular changes is the transition of the pollen grain into a metabolic inactive structure (mature pollen) by extensive dehydration. The cytoplasm of the mature pollen grain consists of stored metabolites that are required for pollen germination and pollen tube growth. Among these stored metabolites, a large stock of pre-synthesized tRNA, rRNA, and ribosomes is present (Mascarenhas, 1975; Tupý, 1977; Hoekstra and Bruinsma, 1979).

It is obvious that the course of developmental processes in pollen from various plant species is reflected in the organization of genetic programs that determine gametophytic gene expression. An up-to-date overview about the organization and conservation of gene expression programs during pollen development and pollen tube growth is given in **chapter 1**. Several biochemical experiments with pollen RNAs and proteins and characterization of a large number of pollen-expressed genes from various plant species have provided profound insights in the dynamics of transcriptional and translational processes during pollen development and pollen tube growth. An interesting phenomenon in different plant species is that many abundant mRNAs that are present in the mature pollen grain persist during subsequent pollen germination and pollen tube growth (Mascarenhas, 1975; Frankis and Mascarenhas, 1980; Mascarenhas et al., 1984; Schrauwen et al., 1990). Together with the pre-synthesized tRNA, rRNA and ribosomes, these mRNAs are used for synthesis of proteins that are required for pollen germination and pollen tube growth (Hoekstra and Bruinsma, 1979; Čapková et al., 1988; Mascarenhas, 1989, 1993; Štorchová et al., 1994). An example of a pollen-expressed gene that exhibits such delayed translation of its mRNA is *ntp303* from tobacco (Čapková et al., 1994; Štorchová et al., 1994; Wittink et al., 2000). Delayed translation of pre-synthesized pollen transcripts emphasizes the importance of post-transcriptional processes for the regulation of pollen gene expression.

Translation efficiency of mRNAs is often modulated by sequences that reside in the 5'-untranslated region (5'-UTR; for review, see Gallie, 1993, 1996; Fütterer and Hohn, 1996;

Day and Tuite, 1998; Bailey-Serres, 1999). With regard to this, we assume that the 5'-UTR of stored pollen transcripts plays an important regulatory role in the modulation of gene expression during pollen development and pollen tube growth. The aim of the present thesis was to investigate to what extent the 5'-UTR of pollen-expressed genes plays a role in the regulation of gene expression during pollen development and pollen tube growth. Therefore, the research has been concentrated on two main objectives: functionality of pollen gene 5'-UTR sequences (**chapter 2 and 3**) and identification of putative regulatory pollen gene 5'-UTR sequence elements (**chapter 4 and 5**).

The influence of the *ntp303* UTRs on translation of *ntp303* mRNA during pollen development and pollen tube growth is described in **chapter 2** and **3**. Transient expression experiments using several gene fusion constructs that contain different promoter and UTR combinations revealed that the *ntp303* 5'-UTR enhances translation of chimeric transcripts during pollen tube growth to a level that is much higher than constructs containing different control 5'-UTRs (**chapter 2**). The *ntp303* 3'-UTR did not influenced pollen gene expression. Furthermore, these experiments demonstrated that the 5'-UTR acts as an autonomous enhancer element independent of linked promoter, coding region, or 3'-UTR sequences. A comparison between the relative transcript and reporter protein (luciferase) activity levels revealed that the enhancement is mainly the result of an increase in the translation efficiency and thus of a post-transcriptional regulation event.

A detailed study of the activity of the *ntp303* 5'-UTR during pollen development and pollen tube growth was performed using stably transformed tobacco plants (**chapter 3**). Although transcripts containing the *ntp303* 5'-UTR were translated during pollen development, their translation efficiency increased strongly at the onset of pollen germination. During subsequent pollen tube growth in the style and ovary, the *ntp303* 5-UTR maintained a high and constant level of translation. Examination of the activity of the *ntp303* 5'-UTR in transgenic seed revealed that the UTR mediates efficient translation in rehydrated tissues. These results indicate that processes underlying rehydration act positively on the activity of the *ntp303* 5'-UTR. These processes might represent important cellular signals that direct efficient translation of stored mRNAs via the *ntp303* 5'-UTR. The transgenic tobacco plants allowed us also to detect *ntp303* expression in non-pollen tissues such as the stigma and ovary. *Ntp303* expression in these tissues was confirmed by RT-PCR and was primarily regulated by the *ntp303* promoter.

In order to identify 5'-UTR sequences that are important for the post-transcriptional regulation of *ntp303* translation in pollen tubes, a number of *ntp303* 5'-UTR deletion

constructs were generated and tested (**chapter 2**). Sequence regions that are located in two stem loop structures (H-I and H-II) were shown to be essential for enhancement of translation during pollen tube growth. Measurements of the effect of the different deletions on the transcript accumulation and luciferase activity levels indicated that H-I sequences are important for modulation of translation efficiency, whereas H-II sequences determine transcript stability. Moreover, a $(GAA)_8$ repeat within the H-I structure was shown to be essential for the high level of translation in pollen tubes. In order to examine to what extent the regulatory sequence regions of the *ntp303* 5'-UTR were conserved in the 5'-UTR of *ntp303* homologous genes from other plant species, a petunia homologue (*php303*) was isolated (**chapter 4**). Both *ntp303* and *php303* were highly homologous to *lat51* from tomato, which enabled an extended comparative study with regard to the 5'-UTR sequences. Northern and Western analyses revealed delayed translation of *php303* mRNA during pollen development, indicating that PHP303 protein synthesis is also regulated at the post-transcriptional level. The delay of *php303* mRNA translation differed from that of *ntp303* in such a way that the protein was already synthesized during pollen development. This indicates that variation exists between genetic programs in pollen of closely related plant species. Like the *ntp303* 5'-UTR, the *php303* 5'-UTR was able to enhance gene expression in pollen tubes. Alignment of the 5'-UTRs of *ntp303*, *php303*, and *lat51* revealed the presence of several conserved sequence regions, including those that have been shown previously to direct efficient translation of *ntp303* transcripts. The similar expression characteristics, functionality and composition of 5'-UTR sequences of pollen-expressed genes from closely related species clearly argue for an evolutionary conservation of regulatory mechanisms that direct gene expression in pollen.

In order to identify putative regulatory sequence elements in heterologous 5'-UTR sequences of pollen-expressed genes, a statistical-based computer analysis has been carried out (**chapter 5**). Analysis of 5'-UTR sequences of pollen-expressed genes and genes that are expressed in sporophytic tissues demonstrated that a large number of sequence elements are preferential present in the 5'-UTR of pollen genes. The pollen sequence elements were randomly distributed in the 5'-UTRs in a relative low copy number. Several of the over-represented sequence elements were also present in the previously defined functional regions of the *ntp303* 5'-UTR, which indicates that these sequences resemble regulatory elements. This accounts clearly for the sequence element AAGAAG, which has been previously demonstrated to be important for enhancement of translation of *ntp303* transcripts. Remarkably, several pollen sequence elements were significantly over-represented in the 5'-

UTR of genes that are derived from dicotyledonous plants, plants containing wet-type stigmas, or plants containing bicellular pollen.

The presence of several populations of mRNAs that differ in their course of accumulation and degree of translation indicates that regulation of pollen gene expression comprises a coordinated mode of action of regulation mechanisms that act both at the transcriptional and post-transcriptional level. The overall presence of these mechanisms in pollen from different plant species argues for an evolutionary conservation of genetic programs that determine development and germination of pollen. At the molecular level, the extent of conservation of these genetic programs is clearly reflected in the presence of sequence elements that are over-represented in the 5'-UTR of pollen-expressed genes which code for a variety of products. Concerning the mRNAs that are present in the mature pollen grain, it is obvious that the various genetic programs reflect the variation in translatability of the different transcripts. An intriguing idea is that the conserved sequence elements might be involved in the implementation of these different genetic programs by means of an interaction with regulatory molecules like proteins or complementary RNAs.

In a first attempt to identify these regulatory factors, we have carried out a phage display assay using different *ntp303* RNA probes and phage display libraries from mature and germinating pollen (R.J.M. Hulzink, unpublished data). Unfortunately, we have not succeeded yet in the identification of proteins that specifically bind to regions of *ntp303* transcripts. However, application of the different *ntp303* RNA probes in RNA hybridization experiments revealed an unexpected interaction of sense *ntp303* mRNA with unknown RNAs of 2100 and 4500 base pairs in length (R.J.M. Hulzink, unpublished data). RNA:RNA interaction is not an unusual phenomenon in animal and plant systems and is thought to be of importance for regulation of gene expression (for review, see Klaff et al., 1996; Dolnick, 1997; Vanhée-Brossollet and Vaquero, 1998; Terryn and Rouzé, 2000). These observations provide additional and unexpected clues for the importance of post-transcriptional processes in the regulation of pollen gene expression.

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Chapter 7

Samenvatting en conclusies

Honderd miljoen jaar evolutie van bloeiende planten (angiospermen) heeft geleid tot de aanwezigheid van maar liefst 250000 verschillende plantensoorten. De verscheidenheid aan bloeiende planten komt duidelijk tot uiting in de grote variatie aan bestuivings- en bevruchtingssystemen. Ondanks de variatie van bloeiende planten verlopen vele reproductieprocessen min of meer hetzelfde. Een voorbeeld hiervan is de ontwikkeling van mannelijke gametofyten oftewel stuifmeelkorrels (pollen). De ontwikkeling van stuifmeelkorrels van monocotyle en dicotyle plantensoorten omvat verschillende cytologische veranderingen (Mascarenhas, 1989; Scott et al., 1991; Bedinger, 1992; Goldberg et al., 1993; McCormick, 1993). Eén van de meest opvallende cytologische veranderingen is de sterke daling van het vochtgehalte van pollen tijdens de ontwikkeling. Door deze dehydratatie veranderen pollen in metabolisch inactieve structuren die een grote hoeveelheid opgeslagen metabolieten, tRNAs, rRNAs en ribosomen bevatten (Mascarenhas, 1975; Tupý, 1977).

Het is vanzelfsprekend dat het verloop van de ontwikkelingsprocessen in pollen zijn basis heeft in de organisatie van de genetische programma's die de gametofytische genexpressie bepalen. Een actueel overzicht van de genexpressie tijdens de pollenontwikkeling en pollenbuisgroei is beschreven in **hoofdstuk 1**. Biochemische experimenten met pollen RNAs en eiwitten en de karakterisering van pollen-"specifieke" genen uit verschillende plantensoorten hebben veel inzicht gegeven in het verloop en de organisatie van transcriptionele en translationele processen. Een interessant fenomeen dat voorkomt bij verschillende plantensoorten is de opslag van een grote hoeveelheid mRNAs (Mascarenhas, 1975; Frankis and Mascarenhas, 1980; Mascarenhas et al., 1984; Schrauwen et al., 1990). Deze transcripten worden samen met de opgeslagen tRNAs, rRNAs en ribosomen gebruikt voor de synthese van eiwitten die nodig zijn voor pollenkieming en pollenbuisgroei (Hoekstra and Bruinsma, 1979; Čapková et al., 1988; Mascarenhas, 1989, 1993; Štorchová et al., 1994). Een voorbeeld van een pollen-"specifiek" gen waarvan het mRNA wordt opgeslagen is *ntp303* uit tabak (Čapková et al., 1994; Štorchová et al., 1994; Wittink et al., 2000). Uitgestelde translatie van opgeslagen mRNAs benadrukt het belang van post-transcriptionele processen voor de regulatie van genexpressie in pollen.

De efficiëntie van de mRNA translatie wordt onder andere bepaald door sequenties in het 5'-ongetransleerde gebied (aangegeven als "5'-untranslated region" of "5'-UTR"; Gallie, 1993; Fütterer and Hohn, 1996; Gallie, 1996; Day and Tuite, 1998; Bailey-Serres 1999). Daarom veronderstellen wij dat het 5'-UTR van opgeslagen pollentranscripten een belangrijke regulerende rol speelt in de modulatie van genexpressie tijdens

pollenontwikkeling en pollenbuisgroei. Het doel van dit promotieonderzoek was dan ook om te onderzoeken in hoeverre het 5'-UTR van pollen-"specifieke" genen een rol speelt in de regulatie van genexpressie tijdens pollenontwikkeling en pollenbuisgroei. Daarbij heeft het onderzoek zich gericht op twee belangrijke punten: functionaliteit van het 5'-UTR van pollentranscripten (**hoofdstuk 2 en 3**) en identificatie van potentiële regulerende 5'-UTR sequentie-elementen in pollentranscripten (**hoofdstuk 4 en 5**).

De invloed van de UTRs van het *ntp303* gen op translatie van *ntp303* mRNA tijdens pollenontwikkeling en pollenbuisgroei is beschreven in **hoofdstuk 2 en 3**. "Transiente" expressie-experimenten met genfusieconstructen toonden aan dat transcripten met het *ntp303* 5'-UTR een hoger translatieniveau geven dan transcripten die controle 5'-UTRs bevatten (**hoofdstuk 2**). Het *ntp303* 3'-UTR had geen invloed op het translatieniveau. Verder bleek uit de experimenten dat de activiteit van het *ntp303* 5'-UTR onafhankelijk is van promoter-, coderend gebied- en 3'-UTR-sequenties. Een vergelijking tussen de transcriptaccumulatie- en eiwitactiviteitniveaus toonde aan dat het hoge expressieniveau van *ntp303* 5'-UTR-mRNA het resultaat is van een efficiënte translatie en dus van een post-transcriptioneel proces. Vervolgens werd een studie uitgevoerd naar de activiteit van het *ntp303* 5'-UTR tijdens de pollenontwikkeling en pollenbuisgroei. Hierbij werd gebruik gemaakt van getransformeerde tabaksplanten (**hoofdstuk 3**). In tegenstelling tot kiemende pollen werden transcripten die het *ntp303* 5'-UTR bevatten op een lager niveau getransleerd tijdens de pollenontwikkeling. Tijdens de pollenbuisgroei in de stijl en in het ovarium zorgde het *ntp303* 5'-UTR voor een hoog en constant translatieniveau. Onderzoek naar de activiteit van het *ntp303* 5'-UTR in transgeen zaad toonde aan dat het UTR een efficiënte translatie geeft in rehydraterend weefsel. Deze resultaten geven aan dat cellulaire processen, die zich afspelen tijdens rehydratatie, positieve signalen bevatten voor de activiteit van het *ntp303* 5'-UTR. Met behulp van de transgene tabaksplanten kon ook worden aangetoond dat de expressie van het *ntp303*-gen niet beperkt is tot pollen, maar ook plaatsvindt in de stempel en het ovarium. Dit werd bevestigd met RT-PCR. *Ntp303* expressie in de vrouwelijke voortplantingsorganen wordt primair geregeld door de *ntp303* promoter.

Om *ntp303* 5'-UTR sequentie-elementen te identificeren die een rol spelen bij de efficiënte translatie van *ntp303* mRNA tijdens de pollenbuisgroei werd een serie 5'-UTR-deletieconstructen gemaakt (**hoofdstuk 2**). De 5'-UTR-deletieconstructen leidden tot de identificatie van sequentiegebieden in twee secundaire RNA-structuren (H-I en H-II) die na deletie een afname lieten zien van *ntp303* mRNA translatie. Een bepaling van het transcriptaccumulatieniveau van de verschillende deletieconstructen toonde aan dat H-I-

sequenties belangrijk zijn voor de modulatie van de translatie-efficiëntie. Ook bleek dat een $(GAA)_8$-element binnen de H-I-structuur essentieel is voor het hoge *ntp303* translatieniveau tijdens pollenbuisgroei. Deleties van de H-II-structuur lieten zien dat H-II-sequenties een rol lijken te spelen in de determinatie van mRNA-stabiliteit. Een *ntp303* homoloog gen uit petunia (*php303*) werd geïsoleerd en gekarakteriseerd om na te gaan in hoeverre de functionele *ntp303* 5'-UTR-sequenties geconserveerd zijn in pollen-"specifieke" genen van andere plantensoorten (**hoofdstuk 4**). "Northern" en "Western"-analyses toonden aan dat, net als *ntp303*, ook *php303* een uitgestelde translatie kent van het mRNA. Echter uitstel van *php303* mRNA translatie verschilde in de tijd met dat van *ntp303* mRNA, wat een aanduiding is voor het bestaan van verschillen tussen genetische programma's in pollen uit gerelateerde plantensoorten. Door middel van een "transiente" expressie-assay werd vervolgens aangetoond dat, net als het 5'-UTR van *ntp303*, ook het *php303* 5'-UTR een hoger expressieniveau geeft dan controle 5'-UTRs. Een onderlinge vergelijking van 5'-UTR sequenties van *ntp303*, *php303* en *lat51* (een *ntp303* homoloog gen uit tomaat) liet zien dat er verschillende 5'-UTR sequentiegebieden bestaan die sterk geconserveerd zijn. Een aantal van deze sequentiegebieden bevatten de functionele H-I- en H-II-sequenties van het *ntp303* 5'-UTR. De vergelijkbare expressie en functionaliteit en samenstelling van het 5'-UTR van pollen-"specifieke" genen uit gerelateerde plantensoorten geven duidelijk aan dat regulatiemechanismen die belangrijk zijn voor genexpressie in pollen evolutionair geconserveerd zijn.

Om potentiële regulerende sequentie-elementen te identificeren, in een verzameling van heterogene 5'-UTR-sequenties van pollen-"specifieke" genen uit verschillende plantensoorten, is een computeranalyse uitgevoerd (**hoofdstuk 5**). Het zoeken naar 5'-UTR-hexanucleotiden, die preferentieel aanwezig zijn in pollen-"specifieke" genen, resulteerde in de identificatie van een groot aantal pollen-"specifieke" sequentie-elementen. Deze sequentie-elementen bevonden zich willekeurig en in kleine aantallen in het 5'-UTR van de pollen-"specifieke" genen. Een aantal pollen-"specifieke" sequentie-elementen waren ook aanwezig in de functionele sequentiegebieden van het *ntp303* 5'-UTR. Deze waarneming suggereert dat dergelijke sequentie-elementen functioneel zijn. Dit geldt zeker voor het sequentie-element AAGAAG, dat van belang is voor translatie van *ntp303* transcripten tijdens de pollenbuisgroei. Opvallend is dat verschillende computergeïdentificeerde sequentie-elementen preferentieel aanwezig zijn in het 5'-UTR van genen die afkomstig zijn van dicotyle plantensoorten, plantensoorten met natte stempels, of plantensoorten met tweecellige pollen.

De aanwezigheid van verschillende mRNA-populaties met specifieke accumulatie en translatiekarakteristieken in pollen duidt aan dat de regulatie van genexpressie in pollen een gecoördineerde activiteit is, waarbij verschillende regulatiemechanismen op zowel transcriptioneel als post-transcriptioneel niveaus werkzaam kunnen zijn. De aanwezigheid van deze mechanismen in pollen van verschillende plantensoorten is een argument voor een evolutionair behoud van genetische programma's die essentieel zijn voor de ontwikkeling en kieming van pollen. Op moleculair niveau is de conservatie van deze genetische programma's duidelijk terug te vinden in de preferentiële aanwezigheid van sequentie-elementen in pollen-"specifieke" genen. Een intrigerend idee is de mogelijke betrokkenheid van geconserveerde pollen-"specifieke" sequentie-elementen bij de implementatie van deze verschillende genetische programma's via interactie met regulerende moleculen zoals eiwitten of complementaire RNAs. "Phage-display"-experimenten zijn uitgevoerd om dergelijke regulerende moleculen te identificeren (R.J.M. Hulzink, ongepubliceerde data). Hierbij is gebruik gemaakt van verschillende *ntp303* RNA probes en faagbanken van volwassen en kiemende pollen. Deze aanpak heeft nog niet geleid tot de identificatie van eiwitten die specifiek aan het 5'-UTR van *ntp303* binden. Het gebruik van verschillende *ntp303* RNA probes in RNA hybridisatie-experimenten heeft echter wel geleid tot de waarneming van een interactie van sense *ntp303* mRNA met RNAs van 2100 en 4500 basenparen in lengte (R.J.M. Hulzink, ongepubliceerde data). RNA:RNA-interactie is geen ongewoon fenomeen in verschillende dierlijke en plantaardige systemen en blijkt een belangrijke rol te spelen in de regulatie van de genexpressie (Klaff et al., 1996; Dolnick, 1997; Vanhée-Brossollet en Vaquero, 1998; Terryn en Rouzé, 2000). Deze waarnemingen geven additionele en onverwachte argumenten om het belang van post-transcriptionele processen voor de regulatie van genexpressie in pollen te onderschrijven.

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Curriculum vitae

De auteur van dit proefschrift werd geboren op 15 maart 1970 te Groenlo. Na de HAVO genoten te hebben aan de scholengemeenschap "Marianum" te Groenlo, begon hij in 1990 aan de opleiding "2e graads leraar biologie" aan de "Educatieve Faculteit Hogeschool Gelderland" in Nijmegen. Tijdens de afstudeerfase van deze opleiding heeft de auteur 3 maanden les gegeven aan de scholengemeenschap "EdeNoord" en 3 maanden onderzoeksstage gelopen bij de afdeling "Aquatische Oecologie" aan de Katholieke Universiteit Nijmegen. Het onderzoek op deze afdeling heeft hem enthousiast gemaakt voor een universitaire studie biologie, waarmee hij in 1994 is begonnen aan de Katholieke Universiteit Nijmegen. Tijdens zijn afstudeerfase heeft de auteur twee hoofdvakstages gelopen. De eerste stage werd verricht op de afdeling "Celbiologie van de Plant" onder leiding van Dr. Wim Vriezen (begeleider) en Prof. Dr. Titti Mariani (hoogleraar). Deze stage heeft geleid tot een onderzoeksverslag met als titel "Structure and expression of cDNAs encoding ACC oxidase homologs isolated from submerged *Rumex palustris* plants". De tweede stage werd verricht op de afdeling "Ontwikkelingsbiologie" van het CPRO-DLO te Wageningen onder leiding van Dr. Martin Kater (begeleider), Prof. Dr. Gerco Angenent (begeleider) en Prof. Dr. George Wullems (hoogleraar). Dit heeft geresulteerd in een onderzoeksverslag met als titel "Cloning and characterization of a class IV chitinase from *Brassica napus* which is up-regulated during early stages of silique development". In aanlsuiting op de stages heeft de auteur twee hoofdvakscripties geschreven met als titel "1-(malonylamino)cyclopropane-1-carboxylic acid: a major metabolite of the ethylene precursor 1-cyclopropane-1-carboxylic acid in higher plants" en "Apoptosis: a case of conservation. Determination of apoptosis in higher plants". Op 27 januari 1998 is de auteur afgestudeerd in de richting "Algemeen Biologisch en Oecologisch", waarna hij op 1 februari 1998 in dienst trad als AIO'er bij de afdeling "Moleculaire Plantenfysiologie" van de Katholieke Universiteit Nijmegen. Op deze afdeling, onder leiding van Prof. Dr. George Wullems, heeft hij vier jaar onderzoek verricht naar de functie van "5'-untranslated regions" voor de regulatie van genexpressie in pollen. Daarnaast heeft hij zeven studenten begeleid bij hun doctoraal- of afstudeerstage en heeft hij drie keer geassisteerd bij het derdejaars practicum "Biotechnologie van Plant en Micro-organisme". Verder heeft hij tijdens zijn promotieonderzoek lezingen en presentaties gegeven in Leicester (Engeland), Banff (Canada), Wageningen, Lunteren en Nijmegen.

DANKWOORD

Na vier jaar onderzoek en een half jaar schrijfwerk wordt het nu dan toch echt tijd om het best gelezen deel van het proefschrift te schrijven, kortom het dankwoord. Een promotieonderzoek uitvoeren doe je niet alleen; veel mensen zijn er bewust of onbewust bij betrokken en hebben daarom, op welke wijze dan ook, hun (edel)steentje bijgedragen aan een product waar ik trots op ben, mijn onderzoek. Dit onderzoek is op het meest basale niveau niet mogelijk geweest door de inbreng (de willig- en onwilligheid) van mijn modelsysteem, *ntp303*. Verguisd door kortzichtigen en begrepen door denkers heeft het gen ons door de eeuwen heen waardevol inzicht verschaft in de verbazingwekkende fysiologie van ontwikkelende en kiemende stuifmeelkorrels (dit lezen jullie goed heren plantenfysiologen). Echter uitzicht op de thuishaven van ultiem inzicht is alleen maar ongrijpbaarder geworden; een wetmatigheid dat ook voor mijn onderzoek geldt.

Toen ik in de vorige eeuw mijn nieuwe werkplek binnenstapte, het lab UL134, ontdekte ik al snel dat ik, in die bijzondere volgestouwde ruimte met al dat prachtige en met veel vakwerk geblazen glaswerk, niet alleen was. Al gauw bleek een monotoon geluid dat zich ergens uit een hokje in het lab ontsproot, een uiting te zijn van communicatie van een organisme wat zich later manifesteerde als een groot denker, een groot kenner der chemie, een veel te fanatieke sportbeoefenaar, een twijfelaar, een trotse papa, een schuine humoraanhanger en een goed mens. Vanaf dat moment heeft deze persoon, oftewel Peter (de lip) is zijn naam (en praten is zijn faam), een belangrijke rol gespeeld binnen mijn onderzoek door er onder andere voor te zorgen dat, naast het volkomen lamleggen van werkzaamheden door cyclische gespreksvoering, essentiële proeven en werkzaamheden met een grote vakkundigheid werden uitgevoerd. Mijn oprechte dank hiervoor! Hierbij bied ik je aan om volgend voorjaar weer met z'n tweeën (en in korte broek) aan een touw te gaan hangen.

Niet ver verwijderd van dit lab en alleen te bereiken via een lange, donkere, soms natte tunnel (waar denken we aan?) bevind zich een ruimte dat zich het beste laat omschrijven als de werkplaats van kapitein Theo. Naast het betere kluswerk aan zijn berenboot, het ontdekken van de mogelijkheden van het internet (big brother is watching you!), het focussen van zijn zoomlens op alles wat maar beweegt (en dan voornamelijk synchroon beweegt) en het zich laten opsluiten door achtpotigen, heeft kapitein Theo zich heel nuttig beziggehouden met een scala aan belangrijke "onderzoeksondersteunende" klussen. Dankjewel! Ik word je scheepsmaatje zogauw jij het anker van de berenboot in Monaco over boord gooit.

Een ander bijzonder mens die in de vorige eeuw in dezelfde gang was gezeteld is Wim. Denderden mijn kegels en staafjes nog na van het aanzicht van UL134, zo kwamen ze tot rust in het lab van Wim. Waren mijn hersenen nog vertroebeld door de onkuise moppen van Theo, zo kwamen ze tot rust en werden ze tot logisch nadenken geprikkeld bij Wim; een groot denker en een zorgvuldig doener. Oprechte dank voor je waardevolle aanwezigheid en inbreng binnen mijn onderzoek. En uiteraard dank voor "Canto ostinato", "Lemniscaat" en "Palimpsest". We hebben samen nog een concert tegoed!

Naast UL134 is de voormalige werkkamer van een heel opmerkelijk persoon gesitueerd, die van mijn copromotor Rinus. Rinus is een bijzonder mens met het uithoudingsvermogen van een Fries werkpaard, het gemoedelijke en sociale karakter van een echte Broabander, en een bijzondere relaxte manier van autorijden (bijna liggend en al pratend sturen, waarbij zowel het spitsverkeer in Brussel als de inzittende niet uit het oog worden verloren). Als mens, copromotor en wetenschapper heeft hij diep respect bij mij afgedwongen. Ik denk met veel plezier terug aan de lange discussies en brainstormsessies die we met ons tweeën hebben gevoerd, jou inleidende verhalen tijdens (werk)besprekingen, de spanning tijdens onze eerste manuscriptsubmissie, en jou periodieke "regel"buien. Wat de toekomst ook brengt, ik hoop dat we contact blijven houden (is het alleen al vanwege de culinaire hoogstandjes van Joke).

Ongeveer halverwege mijn AIO-periode zijn Rinus, Peter, Wim en ik verhuisd naar "de gang aan de andere kant van de klapdeuren". Hier is onder andere George mijn promotor gevestigd. Voordat ik AIO'er werd op de afdeling heb ik een hoofdvakstage gelopen via en een scriptie geschreven voor de afdeling van George. Mede door zijn persoonlijke aandacht tijdens deze periode, hoefde ik niet lang na te denken over het aanbod om AIO'er te worden bij moleculaire plantenfysiologie. Gedurende dit AIO-schap heeft George mij alle vrijheid gegeven om het onderzoek met plezier uit te voeren. Mijn dank hiervoor!
Naast de kamer van George bevindt zich "het hokje" van José. Op papier is ze de secretaresse van de afdeling, maar in de praktijk veel meer dan dat. Onder het genot van een wortelsapje en vaak onderbroken door een lachsalvo, kon José heerlijk vertellen over haar strandjutterervaringen (de ene schelp is de andere niet, neeee) en natuurlijk over de hond. De tussen het lachen door vertelde verhalen van José waren altijd een goede afleiding voor de AIO'er om eens lekker los te komen van PCR-, kloneer-, gedegradeerd polyribosomaal-geassocieerd RNA-, genomewalking- en phagedisplayproblemen (om maar eens een kleine selectie te noemen van werkzaamheden die niet terug te vinden zijn in dit proefschrift). Bedankt voor deze ontspannende momenten!
Een ander aanstekelijk lachje kwam wel eens uit een kamertje dat tegenover het kantoortje van José is gevestigd, de kamer van Ton (en later ook van onze mobiele Rinus). Ton heb ik leren waarderen als een inspirerende wetenschapper. Een man die de plantenfysiologie een warm hart toedraagt, maar die zich ook niet uit het veld laat slaan door de moderne moleculaire biologie (".... waarin controles en significanties vaak ver te zoeken zijn...."). Het belangrijkste stuk gereedschap van Ton tijdens het bedrijven van wetenschap en onderwijs was zondermeer zijn enthousiasme. Echter voor het redigeren van manuscripten was hij aangewezen op zijn beroemde (beruchte) rode pen (met een oneindige hoeveelheid inkt) en wanneer moderne muziekklanken zijn gehoor binnendrongen hanteerde hij met veel kunde z'n rechterarm om labdeuren te sluiten of radio's het zwijgen op te leggen. Desondanks, bedankt voor je waardevolle inbreng binnen mijn onderzoek!
Prettige herinneringen heb ik ook overgehouden aan één van de laatste plantenfysiologische AIO'ers die nog op de afdeling (wereld) rondliep. Verstopt in een oerwoud van zich tegen onzichtbare draadjes en ramen omhoog woekerende planten, omgeven door zwaartekrachttartende stapels papieren, gezeteld onder een paraplu en papieren vliegtuigjes en gadegeslagen door een drietal onregelmatig vibrerende plastic visjes in een aquarium dat zich aquaworld durfde te noemen, heeft deze AIO'er, ook wel Marc (met een c) of Sonny genaamd, mij ingewijd in de wereld van de harde plantenfysiologie (met als belangrijkste apparaat het "smartsysteem") en in de nog hardere wereld van de gegevensverwerking- en analyse (met als belangrijkste apparaat de pc). Vooral in dat laatste konden we ons beiden erg goed vinden. Momenten waarop andere mensen zich distantieerden van het arbeidsproces middels pauzes, werden door ons aangewend om ons verder te verdiepen in moeizame, ingewikkelde en soms onvoorspelbare interacties tussen .... euch ..... wormen en cowboys ... uhm! Gelukkig worden deze moeizame werkzaamheden tot op heden voortgezet in Apeldoorn onder de noemer "werkbespreking"; een goede traditie die zeker voortgezet dient te worden (is het alleen maar om een keertje te .... euch ....winnen). Marc, to(t) battle!
Uiteraard is mijn onderzoek niet mogelijk (en soms onmogelijk) geweest zonder de inbreng van een aantal studenten. William, Han, Saskia, Jaap, Laura, en Lydia, hartstikke bedankt voor jullie aandeel! Een aantal experimenten zijn niet mogelijk geweest zonder de hulp van een aantal mensen van buitenaf. Sander van der Krol zou ik graag willen bedanken voor zijn waardevolle aandeel in de analyse van de transgene planten. I would like to thank Jacques van Helden for his valuable contribution to the bioinformatic part of my research. Uiteraard dank aan Jelle, Rien en Liesbeth van het gemeenschappelijk instrumentarium die met woord en

daad mijn onderzoek hebben vereenvoudigd. Verder ook dank aan de mensen die echt verstand hebben van planten: Theo, Walter, Harry, Gerard en mijn dorpsgenootje Yvette.
De tien uur koffiepauze was het moment bij uitstek om los te komen van de dagelijkse beslommeringen in het lab. Ondersteund door de bulderende lach van Bart kwamen de stoerste sportverhalen (Huub en Peter), de wildste trektochtenstories (Mieke en Clementine), de sterkste staaltjes aan feitenopsommingen (Jan en Wim), de grootste verhalen uit het UL-verleden (Rinus et al.) en de schuinste anekdotes (Theo et al.) boven tafel. Ontspannende momenten om niet te vergeten! Op zoek naar antwoorden, materialen, hulp of gewoon een praatje kon altijd worden aangeklopt bij "de jongens" (en dames) van Titti en natuurlijk ook bij Titti zelf. Bedankt voor jullie support!
Aan het einde van mijn AIO-periode is een nieuwe onderzoeksafdeling in het leven geroepen onder leiding van Tom. Onder het motto van "één gen maakt nog geen genoom" ("en een genoom maakt nog geen plant"), werden de meest geavanceerde en plezante apparaten de afdeling binnengesleept door een tweetal Belgische mannekes en een Duitse Belgische uit Nederland. Filip, Stefan en Janny, bedankt voor jullie gezelligheid. Gent staat in ieder geval in de agenda! Yoo Tom! Hartstikke bedankt voor je frisse en vergelijkende kijk op de zaken van alledag en je tomeloze inzet voor het nakijken van mijn manuscripten.
Aan het einde van dit dankwoord mogen uiteraard niet de mensen vergeten worden die op bewuste en onbewuste wijze voor de nodige steun hebben gezorgd buiten het lab. Hierbij denk ik aan familie- en schoonfamilieleden, vrienden en natuurlijk mijn schoonouders. Bijzondere dank ook aan mijn lieve ouders voor al hun aandacht. En dan uiteraard dank aan Ilse, mijn lieve vrouw, die absoluut de belangrijkste bijdrage heeft geleverd aan het tot stand komen van mijn onderzoek en het proefschrift. Zonder haar zou het manuscript behoorlijk gedateerd naar de drukker zijn gegaan.